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(71) Applicant *(for all designated States except US)*: THE GATSBY CHARITABLE FOUNDATION [GB/GB]; 9 Red Lion Court, London EC4A 3EB (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JONES, Jonathan, Dallas, George [GB/GB]; 19 Waverley Road, Norwich, Norfolk NR4 6SG (GB). HAMMOND-KOSACK, Kim, Elizabeth [GB/GB]; 6 Chestnut Hill, Norwich RN4 6NL (GB). JONES, David, Allen [GB/GB]; 139 Greenways, Eaton, Norwich NR4 6PD (GB).

(74) Agents: WALTON, Seán, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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(54) Title: METHOD OF INTRODUCING PATHOGEN RESISTANCE IN PLANTS

(57) Abstract

Variegated plants have increased pathogen resistance: cells of the plant express a phenotype, which may comprise necrosis and/or a plant defence response, and other cells not expressing this phenotype have increased pathogen resistance. Embodiments of the invention employ various genes, including *Cladosporium fulvum* pathogen resistance genes, which are inactivated, for example as a result of insertion of a transposable genetic element, and then reactivated in plant cells to result in necrosis and/or a plant defence response, leading to increased pathogen resistance. Cells, plants and other compositions of matter are provided comprising various combinations of genes involved in this system.

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METHOD OF INTRODUCING PATHOGEN RESISTANCE IN PLANTS

The present invention relates to a method of introducing pathogen resistance in plants, particularly broad spectrum pathogen resistance, and plants which may be obtained by said method and which show resistance to at least one but preferably more than one pathogen.

Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, because they are usually grown as genetically uniform monocultures; when disease strikes, losses can be severe. However, most plants are resistant to most plant pathogens. To defend themselves, plants have evolved an array of both preexisting and inducible defences which include barriers to pathogen entry such as thickened or chemically crosslinked cell wall components or toxic chemicals derived from complex plant biosynthetic pathways. Pathogens must specialize to circumvent the defence mechanisms of the host, especially those biotrophic pathogens that derive their nutrition from an intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to be compatible, but if the plant is resistant, the interaction is said to be incompatible.

Induced resistance is strongly correlated with the hypersensitive response (HR), an induced response

associated with localized cell death at sites of attempted pathogen ingress. It is hypothesized that by HR the plant deprives the pathogen of living host cells but there is no certainty about whether localised cell death results from or induces plant defence mechanisms.

Many plant defence mechanisms are strongly induced in response to a challenge by an unsuccessful pathogen. Such an induction of enhanced resistance can be systemic (hereinafter referred to as systemic acquired resistance (SAR)) (Ross, 1961; Ryals *et al.*, 1992). Acquired resistance can also be local (hereinafter referred to as LAR) (Ryals *et al.*, 1992). Acquired resistance has been extensively researched and various facts have been established. For example, biotic stimuli are required to provoke the HR resulting in areas of dead plant cells on the leaf. Cell death resulting from wounding or other abiotic stresses will not suffice. (Ryals *et al.*, 1992; Enyedi *et al.*, 1992). In addition, SAR is correlated with the induction of a large array of pathogenesis-related (PR) proteins, some of which have demonstrated anti-fungal activity (Ward *et al.*, 1991).

A variety of examples of SAR have been studied and include challenging of tobacco carrying the N gene for resistance to tobacco mosaic virus (TMV) with TMV (Ross, 1961) and challenging cucumber seedlings with tobacco necrosis virus or *Colletotrichum largenarium*.

Results show that a challenge with one pathogen leads to enhanced resistance to a wide variety of other pathogens (Ryals *et al.*, 1992).

SAR has also been correlated with increased levels of salicylic acid in plants which have been challenged by pathogens (Malamy *et al.*, 1990; Metraux *et al.*, 1990) which has been confirmed by studies that show that a supply of exogenous salicylic acid to unchallenged plants can result in SAR (Ward *et al.*, 1991; Hennig *et al.*, 1993). Transgenic plants designed so that salicylic acid accumulation is prevented by expression of a salicylate hydroxylase gene show reduced SAR compared to non-transgenic plants where salicylic acid accumulation is not prevented (Gaffney *et al.*, 1993). SAR can also be induced by many chemicals manufactured by Ciba-Geigy such as 2,6-dichloroisonicotinic acid (INA) (Uknes *et al.*, 1992).

SAR is an attractive method by which broad spectrum disease control can be achieved. However, two major drawbacks hinder its commercial exploitation: SAR is not a heritable trait and so the phenomenon has to be successfully induced into every plant in the crop stand; to be effective throughout the crop's life, the SAR phenotype has to be re-boosted at regular intervals.

Although the mechanisms causing SAR are not fully understood, it is believed that when a plant is

challenged by a pathogen to which it is resistant, it undergoes an HR at the site of attempted ingress of the incompatible pathogen. The induced HR leads to a systemic enhancement and acquisition of plant resistance to virulent pathogens that would normally cause disease in the unchallenged plant.

It has long been known that HR-associated disease resistance is often (though not exclusively) specified by dominant genes (R genes). Flor showed that when pathogens mutate to overcome such R genes, these mutations are recessive. Flor concluded that for an R gene to function, there must also be a corresponding gene in the pathogen, an "avirulence gene" (*Avr* gene). To become virulent, pathogens must thus stop making a product that activates R gene-dependent defence mechanisms (Flor, 1971). A broadly accepted working hypothesis, often termed the elicitor/receptor model, is that R genes encode products that enable plants to detect the presence of pathogens, provided said pathogens carry the corresponding AVR gene (Gabriel and Rolfe, 1990). This recognition is then transduced into the activation of a defence response.

The mlo allele of the Mlo gene of barley is the one example of a recessive disease resistance gene currently widely used in plant breeding. Lines that are homozygous for the recessive allele of this gene activate the defence response (comprising formation of

cell wall appositions) even in the absence of the pathogen (Wolter *et al*, 1993). Thus the mlo mutation causes a defence mimic phenotype, also known as a necrotic or disease lesion mimic phenotype, and appears to deregulate the defence response, so that it is activated precociously, or is regulated on more of a "hair trigger". A number of examples of such disease lesion mimic mutants exist in maize (Johal *et al*, 1994, Pryor, 1987, Walbot, 1983). Recently, such mutants have been sought in Arabidopsis. The characterization of one such mutant, acd1, has been reported (Greenberg and Ausubel, 1993). Further mutants of this type have been reported at scientific meetings (the Arabidopsis acd2 mutation by F.M. Ausubel at a meeting at Rutgers University, New Jersey, USA, April 1993; Arabidopsis mutations now known as lsd (for lesions simulating defence response) mutations by R. Dietrich and J. Dangl at the ARAPANET ((Arabidopsis Pathology Network) workshop in Wye College, Kent, UK in April 1993). Manuscripts describing the acd2 and lsd mutations are Dietrich et al. and Greenberg et al. (1994). It is highly likely that the recessive mutations identified in such mutant screens that leave the defence response more constitutively on, or more rapidly activated, or less easily inactivated, are in genes that normally dampen down the defence response to prevent it becoming so severe that it is deleterious to the plant.

Conceivably, such gene could be cloned, expressed in an antisense or sense configuration to reduce expression of the corresponding gene (Hamilton, 1990, Napoli *et al*, 1989).

Pathogen avirulence genes are still poorly understood. Several bacterial *Avr* genes encode hydrophilic proteins with no homology to other classes of protein, while others carry repeating units whose number can be modified to change the range of plants on which they exhibit avirulence (Keen, 1992; Long and Staskawicz, 1993). Additional bacterial genes (*hrp* genes) are required for bacterial *Avr* genes to induce HR, and also for pathogenicity (Keen, 1992; Long and Staskawicz, 1993). It is not clear why pathogens make products that enable the plant to detect them. It is widely believed that certain easily discarded *Avr* genes contribute to but are not required for pathogenicity, whereas other *Avr* genes are less dispensable (Keen, 1992; Long and Staskawicz, 1993). The characterization of two fungal avirulence genes, *Avr 4* and *Avr 9* (De Wit *et al.*, 1992; Joosten *et al.*, 1994), has also been reported. Research is also being undertaken to clone rice blast avirulence genes from the causal organism *Magnaporthe grisea* and the avirulence genes (NIP proteins) of the barley pathogen *Rhynchosporium secalis*. Two viral avirulence genes have also previously been cloned. Culver and Dawson, 1991, have

shown that tobacco mosaic virus coat protein is the avirulence determinant for the N' gene product. In addition, the potato virus X coat protein appears to be the avirulence determinant for the Rx and Nx genes (Kavanagh *et al.*, 1992; Santa-Cruz *et al.*, 1993; Köhm *et al.*, 1993; Goulden *et al.*, 1993).

Recently the map based cloning of the tomato *Pto* gene that confers "gene-for-gene" resistance to the bacterial speck pathogen *Pseudomonas syringae pv* tomato (Pst) has been reported (Martin *et al.*, 1993). It has also been recently reported that the *Arabidopsis* Rps2 gene (which confers *Pseudomonas syringae* resistance) and the tobacco N gene (which confers virus resistance) have been cloned (Keystone Symposium, January 1994). Even more recently, the Rps2 and features of the *Cf*-9 gene sequences have been revealed at the 13th Annual Symposium in Columbia, Missouri, April 13th-16th 1994, on the Biology of Communication in Plants. International Patent Application No: PCT/GB94/02812 describes a method for generally identifying and cloning plant resistance genes.

The technology for gene isolation based primarily on genetic criteria has improved dramatically in recent years, and many workers are currently attempting to clone a variety of R genes. Targets include (amongst others) rust resistance genes in maize, *Antirrhinum* and flax (by transposon tagging); downy mildew resistance

genes in lettuce and *Arabidopsis* (by map based cloning and T-DNA tagging); *Cladosporium fulvum* (Cf) resistance genes in tomato (by tagging, map based cloning and affinity labelling with avirulence gene products); virus resistance genes in tomato and tobacco (by map based cloning and tagging); nematode resistance genes in tomato (by map based cloning); and genes for resistance to bacterial pathogens in *Arabidopsis* and tomato (by map based cloning).

Tomato (*Lycopersicon esculentum*) is susceptible to disease caused by the leaf mould fungal pathogen *Cladosporium fulvum*. According to De Wit, 1992, the *Avr9* gene of *C. fulvum*, which confers avirulence on *C. fulvum* races that attempt to attack tomato varieties that carry the *Cf*-9 gene, encodes a secreted cysteine-rich peptide with a final processed size of 28 amino acids. However, its role in compatible interactions is not clear. The R genes (*Cf*-genes) that act against *C. fulvum* have been identified and bred into cultivated varieties, often from related species of tomato (Dickinson *et al.*, 1993; Jones *et al.*, 1993).

It has been shown that *C. fulvum* contains *Avr* genes that confer recognition by plants which contain the *Cf*-genes , leading to activation of host defence mechanisms to attack the disease (incompatibility). The *Avr4* and *Avr9* genes encode small peptides that are secreted by the pathogen into the intercellular spaces

of infected leaves, from which they can be extracted. This has enabled the purification and sequencing of these peptides and the isolation of the genes that encode them (De Wit, 1992; Joosten *et al.*, 1994). Experiments have shown that when the *Avr9* gene is transformed into a race of pathogen that lacks *Avr9*, then the race of pathogen becomes avirulent on plants which are carrying the *Cf-9* gene. In addition, it has been shown that disruption of the *Avr9* gene in a pathogen race which is avirulent on plants carrying *Cf-9* gene confers compatibility on the *Cf-9* containing plants (Van Den Ackerveken *et al.*, 1992, Marmeisse *et al.*, 1993).

In addition, De Wit and colleagues have further shown that the secreted peptide encoded by the *Avr9* gene can be injected into *Cf-9* containing tomato leaves to elicit a necrotic response in the injected area. The necrotic response is consistent with local and vigorous activation of a defence response (De Wit, 1992; WO 91/15585). International Patent Application No. PCT/GB94/02812 describes the transgenic expression of the *Avr9* gene using the strong cauliflower mosaic virus 355 plant promoter to cause lethality in *Cf-9* plants. This transgenic expression has been used to select mutants in which the *Cf-9* gene has been inactivated by transposon insertion in order to isolate the *Cf-9* gene and perform DNA sequence analysis of this

gene.

Various pathogen races that overcome these *Cf*-genes have emerged and are named after the *Cf*-gene which they can overcome. For example, *C. fulvum* race 4 can overcome *Cf*-4; *C. fulvum* race 5 can overcome *Cf*-5 and *C. fulvum* race 2.4.5.9 can overcome *Cf*-2, *Cf*-4, *Cf*-5 and *Cf*-9.

WO 91/15585 describes a hypothetical method whereby if a *Cf*-9 gene and/or an *Avr*9 gene were expressed under the control of a promoter that is induced by a broad range of pathogens, then a general defence response could be induced. However, there is a lack of enabling disclosure regarding which polynucleotide sequences could be used either as the resistance gene or as an actual promoter which would be suitably affected by a broad range of pathogens. A further problem with this proposed method is that necrosis induced by the *Cf*-9 and *Avr*9 gene combination could lead to further induction of *Avr*9 and/or *Cf*-9 leading to spreading of the necrosis and severe reduction in the yield of the plant. This problem may arise since promoters such as promoters for plant defence genes and other genes involved in the defence response such as PR genes (pathogenesis related genes), are induced in both a compatible and an incompatible interaction. Therefore, even if a promoter exists which is effectively induced by a broad range of

pathogens, the method would not be viable unless the promoter is only induced by the appearance of a compatible pathogen. If the defence response provides further induction of the promoter the plant might experience spreading necrosis.

The present invention has resulted from experiments involving transposon tagging of resistance genes, the first one being *Cf-9*. Numerous alleles of the *Cf-9* gene (*Cf-9*Ds*) were isolated that had been inactivated by the maize element *Dissociation* (*Ds*). These inactive *Cf-9*Ds* genes did not give rise to a constitutive and lethal activation of defence mechanisms in response to constitutively expressed *Avr9* transgene (*35S:SP:Avr9*). On backcrossing plants that carried the *Cf-9*Ds* and *35S:SP:Avr9* genes to tomato plants carrying an *Activator* (*Ac*) transposase gene (*sAc*) in the homozygous state but lacking *Cf-9*, a quarter of the resultant progeny carried *sAc*, *35S:SP:Avr9* and *Cf-9*Ds*. These plants showed somatic excision of *Ds* from the *Cf-9*Ds* gene, somatically restoring *Cf-9* function and giving rise to localised activation in cells of plant defence responses due to recognition of the constitutively expressed *Avr-9* peptide. These cells died and gave rise to small necrotic sectors, the plants phenotypically showing variegation for a defence-related necrosis, similar to somatic flecks of necrosis that are associated with the

induction of SAR in plants challenged with necrotising pathogens. Further work showed that plants that variegate for somatic sectors of plant defence response in this way have increased resistance to a range of pathogens.

Thus, a first aspect of the present invention relates to a method of providing pathogen resistance, in particular broad spectrum pathogen resistance, in plants by induction of variegation in which genes are expressed or suppressed resulting in the activation of necrosis. A method according to the present invention comprises: (i) inactivating a nucleotide sequence which contributes to plant cell necrosis or inactivating one or more nucleotide sequences forming part of a combination of nucleotide sequences which contribute to plant cell necrosis; (ii) introducing said nucleotide sequence or sequences into the genome of a plant; and (iii) restoring said nucleotide sequence or sequences to a functional form to yield a level of necrosis resulting in pathogen resistance. The plant cell necrosis is preferably defence-related plant cell necrosis.

A second aspect of the present invention relates to a method of providing pathogen resistance in plants by induction of variegation in which genes are expressed or suppressed resulting in the activation of a plant defence response which comprises: (i)

inactivating a nucleotide sequence which contributes to the plant defence response or inactivating one or more nucleotide sequences forming part of a combination of nucleotide sequences which contribute to the plant defence response; (ii) introducing said nucleotide sequence or sequences into the genome of a plant; and (iii) restoring said inactivated nucleotide sequence or sequences to a functional form to result in pathogen resistance.

The variegation will generally be for somatic sectors. Pathogen resistance will generally be increased compared with wild-type.

The nucleotide sequence or sequences comprise one or more genes. The plant defence response and/or plant cell necrosis occurs on expression of the gene or genes. The defence response and/or plant cell necrosis can be conditional or unconditional on the expression of one or more interacting genes. A substance or a combination of substances may result in increased pathogen resistance. Examples are discussed further below.

For example, the nucleotide sequence or sequences may comprise a gene encoding either a substance which leads to necrosis, e.g. through activation of the plant defence response, or a substance which leads to a plant defence response with no sign of necrosis. For example, the sequence or sequences may comprise a plant

pathogen resistance gene (R), an avirulence gene (*Avr*) or other elicitor or ligand gene (L) of an R gene, or both and R gene and an L gene.

The inactivation of the nucleotide sequence or sequences which contribute to the plant defence response and/or plant cell necrosis is preferably effected by insertion of a transposable genetic element into the nucleotide sequence or one or more of the nucleotide sequences forming a combination of nucleotide sequences. The transposable genetic element is preferably a transposon or a nucleotide sequence flanked by specific nucleotide sequences so that transposon excision gives rise to activation of the plant defence response and/or necrosis. Thus, insertion of a genetic lesion into the nucleotide sequence disrupts the gene to prevent expression of a product able to function in contributing to the plant defence response and/or plant cell necrosis. In the absence of the lesion, e.g. following excision of a transposable element such as a transposon, the gene may be expressed to produce a functional product, i.e. gene function is restored. The lesion may be inserted into the part of the gene coding for the expression product, or may be in a regulatory sequence such as a promoter required for expression of the product.

In this form of the invention, re-activation within the plant is preferably carried out by

restoraration of the inactivated nucleotide sequence or sequences resulting in activation of a plant defence response and/or necrosis. Such restoration may be caused or allowed by culturing of the plant. Where the nucleotide sequence is inactivated by virtue of insertion of a transposable element therein, the plant genome should contain at least one nucleotide sequence coding for a corresponding transposon activation system (for example, comprising a transposase). Alternatively, the inactive form could be flanked by recombinase recognition sequences that are acted on by a site specific recombination system (comprising a specific recombinase) so that recombination activates the inactive form of the gene. Hence, when the inactivated nucleotide sequence or sequences are introduced into the plant genome somatic excision of the transposon or recombination of the nucleotide sequence occurs in some cells leading to activation of the plant defence response and/or necrosis in specific clones of cells.

The number of cells in which restoration of function occurs may vary. As discussed further below, certain measures are available for optimising the system, e.g. by controlling the frequency of spontaneous excision of a transposable element which is caused or allowed upon cultivation of a plant with the requisite nucleotide sequence or sequences within its

genome.

The present invention further provides transgenic plants having increased pathogen resistance obtainable by the method of the present invention, and any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Derivatives of plants are also provided by the present invention. A derivative is any functional unit derived therefrom howsowever achieved (e.g. functional allele of gene made by mutagenesis, recombinant DNA, synthesis, or plant which could not have been produced without the use or manufacture of the plant from which it is derived.)

Transgenic plants in accordance with the present invention may demonstrate increased pathogen resistance since the induced plant defence response and/or necrosis of plant cells may cause other cells, such as adjacent cells, to acquire pathogen resistance. The activation of, for example, a plant resistance gene in a plant cell is inherited by the progeny and descendants of that cell. The expression of this plant resistance gene leads to initiation of the defence response in cells which may eventually lead to the

death of the participating plant cells resulting in an area of plant cell necrosis. So, plants may have variegation for small somatic sectors in which defence-related plant cell necrosis is activated. This response may induce pathogen resistance in other cells. In an alternative, operating on the same general principle, the expression of one or more plant pathogen resistance gene may either lead to initiation of the defence response only resulting in variegation for small somatic sectors in which the plant defence response is activated or of plant cell necrosis which is not related to the plant defence response resulting in variegation for small somatic sectors in which plant cell necrosis is activated.

Hence, the plant may acquire resistance to a broad range of pathogens and not only to the pathogen associated with the gene or genes contributing to necrosis, for example, *C. fulvum* in the case of the *Cf-9/Avr* gene combination. For example, a transgenic tomato plant according to the present invention may demonstrate resistance against a broad range of pathogens such as one or more bacterial plant pathogens (for example, *Xanthomonas campestris, Pseudomonas syringae)*, fungal plant pathogens (for example, *Phytophthora infestans, Fusarium oxysporum, Botrytis cinerea, Verticillium dahliae, Altenaria solani, Rhizoctonia solani*) and viral pathogens (for example,

TMV, PVX, PVY, TSWV). Similarly, other transgenic plants such as transgenic tobacco, *Arabidopsis* and potato plants may display resistance to a large number of major diseases of important crop species such as, Peronospora, Phytophthora, Puccinia, Erysiphe and Botrytis.

Thus, according to a further aspect of the invention there is provided a plant, or any part thereof, which is phenotypically variegated, with clones of cells expressing a first phenotype and other cells expressing a second phenotype which is increased pathogen resistance compared with wild-type. The first phenotype is preferably necrosis and/or a plant defence response phenotype. As discussed, plants variegated by somatic sector for such a phenotype may have enhanced pathogen resistance as a result of a second phenotype in cells, which may be adjacent to the cells with the first phenotype which are necrotic and/or in which a plant defence response is activated. The phenotypic variegation is likely to result from expression in cells with the first phenotype of a gene or gene, or nucleic acid comprising a gene or genes, which contributes to such phenotype, whereas other cells without such phenotype lack such gene expression. As discussed herein, this may result from reactivation of a previously inactivated gene, such as a resistance gene, for example by random excision of a transposable

element such as a transposon.

In a further aspect, the present invention provides a host cell, such as a plant or microbial cell, or a plant comprising at least one such cell, containing (i) nucleic acid encoding one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis, at least one of the nucleotide sequences being reversibly inactivated, for example by insertion of a transposable element such as a transposon, and (ii) nucleic acid encoding a molecule able to reverse the inactivation, such as, in the case of a transposon, a transposase. Thus, the cell may comprise a plant resistance gene or other gene involved in the plant defence response or able to kill a cell when expressed therein (either alone or incombination with one or more sequences, for example in the case of an *R* gene the corresponding elicitor), the gene being inactivated by insertion therein of a transposon, and the cell further comprising a gene encoding a transposase.

In an exemplary embodiment, the genome of the cell comprises the gene *Cf-9*, or a mutant, derivative, variant or allele thereof which retains *Cf-9* function, inactivated by insertion therein of a transposon, the genome also comprising the *Avr-9* gene, or a mutant, derivative, variant or allele thereof which retains *Avr-9* function, and a gene encoding a transposase able

to excise the transposon from the *Cf-9* gene or functional equivalent. Other resistance genes may be employed, as may genes which do not require the presence of an elicitor molecule to cause cell necrosis, as discussed further elsewhere herein.

The cell may comprise the nucleic acid encoding the various genes by virtue of introduction into the cell or an ancestor thereof of the nucleic acid, e.g. by transformation, using any suitable technique available to those skilled in the art. Furthermore, plants which comprise such cells, and seed therefore, may be produced by crossing suitable parents to create a hybrid whose genome contains the required nucleic acid, in accordance with any available plant breeding technique. For example, a parent strain comprising within its genome a plant resistance gene containing a transposon or other inactivating lesion may be crossed with a second strain comprising within its genome a gene encoding the elicitor molecule for the plant resistance gene and a suitable transposase for excision of the transposon. At least a proportion of the hybrid progeny of the parents, i.e. seed or plants grown therefrom, will comprise the required nucleic acid for activation in the plant of, in this example, the plant resistance gene and, following interaction with the elicitor, the plant defence response and/or plant cell necrosis.

Plants according to this aspect of the present invention will be variegated genetically. Clones of cells will have one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis reactivated by removal of the inactivating lesion such as a transposon, so that a first phenotype such as necrosis is shown, while in other cells the sequence or sequences will remain inactivated so these cells will not show the first phenotype.

Within the cell or cells, the nucleic acid may be incorporated within the chromosome. A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, so such decendants should show the desired phenotypic variegation and so may have enhanced pathogen resistance.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

A further aspect of the present invention provides a method of making such a cell involving introduction of nucleic acid (e.g. a vector) comprising

(i) nucleic acid encoding one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis, at least one of the nucleotide sequences being reversibly inactivated, for example by insertion of a transposable element such as a transposon, and/or (ii) nucleic acid encoding a molecule able to reverse the inactivation, such as, in the case of a transposon, a transposase into a plant cell. Introduction of nucleic acid (i) may be accompanied, preceded or followed by introduction of nucleic acid (ii). Such introduction may be followed by recombination between the nucleic acid and the plant cell genome to introduce the sequence of nucleotides into the genome. Descendants of cells into which nucleic acid has been introduced are included within the scope of the present invention.

The level of the plant defence response and/or plant cell necrosis in the small somatic sectors should be sufficient to result in the induction of acquired resistance or the induction of other defence mechanisms. Since this method leads to activation of acquired resistance but is inherited it is referred to as Genetic Acquired Resistance (GAR). Hence, any system which gives rise to a variegation leading to GAR is applicable to the present invention.

Methods and plants etc. according to the present invention are particularly beneficial since the

nucleotide sequence or sequences which contribute to the plant defence response and/or plant cell necrosis, for example the avirulence and plant resistance genes, may be under control of any suitable promoter, such as a constitutive promoter or, in the case of R genes, their own endogenous promoter, or a cell type specific promoter. Furthermore, the restoration of the nucleotide sequence or sequences, for example by the somatic excision of a transposon, gives rise to recurrent and widespread induction of the plant defence response in many small clones of cells throughout the plant, irrespective of whether or not there has been a challenge by pathogen. The resistance conferred on the plant is therefore constitutive and broad.

The present invention may be used for many applications and is suitable for deployment in F1 hybrid seed production system. In such a system, one of the parents should be homozygous, for example, for the transposase or recombinase gene. In addition, in a system where two components are required for inducing the necrosis such as in the *Avr9/Cf-9* gene combination for example, this parent should also be homozygous for the constitutively expressed genes. The other parent should be homozygous for the gene that encodes the non-autonomous inactivation system, such as the transposon or recombinase-recognition sequences. After making a cross between parents of this genetic constitution, on

somatic excision or recombination, the function of the gene or genes which give rise to the defence response and/or plant cell necrosis is restored in somatic sectors in the resulting progeny.

It will be clear to the person skilled in the art that any gene or combination of genes which contributes to variegation for the plant defence response and/or plant cell necrosis may be used in the method of the present invention. Furthermore, any system which gives rise to inactivation of the nucleotide sequence or sequences and subsequent restoration of functional sequence or sequences may be used.

The present invention also provides in further aspects various compositions of matter comprising combinations of nucleotide sequences encoding various substances employed herein. Such combinations of nucleotide sequences which may be introduced into cells in accordance with the present invention follow:

(X): represents a nucleotide sequence with one or more genes of type X

(XY): represents a nucleotide sequence with one or more genes of type X and one ore more genes of type Y etc.

R: receptor gene

L: ligand gene (capable of interacting with the R gene)

I: genetic insert

A: activator of transposition of genetic insert.

R may encode a substance whose presence in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, with I being a genetic insert able to inactivate R and A encoding a substance able to reactivate R inactivated by I:

(1) Any combination of:

1. (R), (I) and (A);
2. (R) and (IA);
3. (I) and (AR); or
4. (A) and (RI);
5. (RIA).

Alternatively, R and L may encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I being a genetic insert able to inactivate R and/or L and A encoding a substance able to reactivate R and/or L inactivated by I:

(2) Any combination of:

1. (R), (L), (I) and (A);
2. (R), (LI) and (A)
3. (R), (LA) and (I)
4. (R), (IA) and (L)
5. (L), (IR) and (A)

6. (L), (AR) and (I)

7. (I), (LR) and (A)

8. (R) and (LIA)

9. (L) and (IAR)

10. (I), and (ARL); or

11. (A) and (RLI);

12. (RLIA)

If genetic insert (I) is coupled with either the R or the L gene, the number of possible combinations will then be

(1): (RI) and (A); or
(RIA)

(2): (RI)(L) and (A)
(R),(LI) and (A)
(RI) and (LA)
(RA) and (LI)
(RLIA)

Also provided by the present invention is a method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, I and A, wherein R encodes a substance whose presence in a plant results in a plant

defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I, comprising crossing plant lines whose genomes comprise any of R, I, A and combinations thereof, to produce the plant or an ancestor thereof.

A further aspect provides a method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, L, I and A, wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I, comprising crossing plant lines whose genomes comprise any of R, L, I, A and combinations thereof, to produce the plant or an ancestor thereof.

Said plant lines may contain nucleic acid comprising any of R, L, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof

Herein, unless context demands otherwise, a "receptor" is a product encoded by a gene capable of interacting with another product, the ligand.

Various embodiments of the present invention are

now described in more detail below, by way of example and not limitation.

Nucleotide Sequence or Sequences contributing to the Plant Defence Response and/or Necrosis

The nucleotide sequence or combination of nucleotide sequences in which at least one of the sequences is inactivated are numerous and may include an engineered allele of a ubiquitin conjugating enzyme (Becker *et al.*, 1993), the CaMV gene VI protein (Takashashi *et al.*, 1989), a viral coat protein in the presence of the appropriate viral resistance gene, for example Tobacco Mosaic Virus Elicitor Coat Protein and the gene N' (Culver and Dawson, 1991), a bacterial harpin protein (Wei *et al.*, 1992; He *et al.*, 1993), the gene N (see e.g. Whitham et al (1994) and a ToMV-Ob gene cloned by Padgett and Beachy (1993), the potato virus X coat protein and its avirulence determinant, (Kavanagh *et al.*, 1992; Santa-Cruz *et al.*, 1993; Köhm *et al.*, 1993; Goulden *et al.*, 1993), *Pto* and *avrPto* (see e.g. Rommens et al., 1995), *RPS2* of *Arabidopsis thaliana* and the avirulence gene *avrRPt2* (Bent et al., Mindrinos et al.), and genes of *Arabidopsis* such as those identified by Greenberg et al. (1994), Dietrich et al., (1994) and Bowling et al., (1994).

Genes coding for substances leading to rapid cell death, such as BARNASE (Mariani *et al.*, 1990) or

diphtheria toxin (Thorsness *et al.*, 1993) may be usable to induce the changes that lead to GAR even though cell death in these latter examples is not caused by activation of the defence response. It is widely believed amongst researchers in this field that cell death arises from local induction of the defence response and that this cell death can activate adjacent cells to give rise to the defence response. However, the precise cause and effect relationship between these events is not clear at the present time. It is also not clear whether the defence response in plants is necessarily coupled to necrosis. Hence, cells may respond to for example the BARNASE-induced death of adjacent cells by activating a wound-inducible defence response, such as that leading to the activation of protease inhibitors or alkaloid biosynthesis (Ryan 1990). Other genes which may be employed in this way include a proton pump such as a bacterial proton pump like the one expressed by Mittler et al (1995) in transgenic tobacco plants.

A preferred example of the present invention is the use of the *Cf-9/Avr9* gene system. This can involve the matching of a transposon inactivated allele of the *Cf-9* gene to constitutive expression of the *Avr9* gene. This system can be replaced by similar combinations of related genes for example the *Avr4* and *Cf-4* gene, sequence provided herein (cloning of *Cf-4* is described

in a co-pending GB application filed simultaneously with the present application); the *Avr*2 and the *Cf*-2 gene, sequence provided herein (cloning of *Cf-2* is described in GB 9506658.5, priority from which is claimed herein); the *Avr*5 and the *Cf*-5 gene, or by cloning resistance genes and corresponding avirulence genes from other systems, such as *RPP5*, sequence provided herein (cloning of *RPP5* is described in GB 9507232.8, priority from which is claimed herein). It certain cases it may be possible to provoke a suitable response in plant cells expressing an R gene in the absence of corresponding *Avr*, for instance by overexpression.

It should also be noted that complete *Avr* or other elicitor gene may not be required. Instead a fragment may be employed, representing a part of the elicitor molecule which interacts to provoke a plant defence response and/or plant cell necrosis.

It is possible that the nucleotide sequence comprises the inactivated R gene, the inactivated *Avr* gene or both, or comprises both the R and *Avr* gene wherein one of the genes is inactivated. Depending of the genes used, the plant defence response and/or plant cell necrosis may be dependent on the expression of both genes and so one example would be that the R gene could be constitutively expressed and the *Avr* gene could exhibit somatic variegation for expression due to

somatic excision and restoration of *Avr9* gene expression, or vice versa.

Nucleotide sequences employed in the present invention may encode a wild-type sequence (e.g. gene) selected from those available, or a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. An alteration to or difference in a nucleotide sequence may or may not be reflected in a change in encoded amino acid sequence, depending on the degeneracy of the genetic code. Preferred mutants, derivatives and alleles are those which retain a functional characteristic of the protein encoded by the wild-type gene, in the present context the ability to contribute to a plant defence response and/or plant cell necrosis. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

Similarly, homologues of the various genes whose use is disclosed herein from other species or races may be employed, as may mutants, variants and derivatives of such homologues.

Inactivation and Reactivation of the nucleotide Sequence or Sequences Contributing to the Plant Defence Response and/or Necrosis

A method according to the present invention may

employ any of a variety of transposon systems known to the skilled person, including the maize Activator/Dissociation (hereinafter referred to *Ac/Ds* system) (Fedoroff, 1989); the maize Enhancer/Suppressor mutator (En/Spm) system (Fedoroff, 1989); and the Antirrhinum Tam1 and Tam3 systems (Coen *et al.*, 1989). In addition, any modified recombination systems which are engineered to yield the appropriate results may be employed, such as, the bacterial Cre-Loxp (Odell *et al*, 1990) or the "FLP/FRT" system (Lloyd and Davis, 1994).

It will be apparent to the skilled person that the particular choice of transposon, recombination or other system used to inactivate the nucleotide sequence or sequences which encode substances leading to the plant defence response and/or plant cell necrosis is not essential to or a limitation of the present invention.

In some systems, a transposon or recombination system might be so active that an unacceptable level of necrosis is seen. If encountered, this may be overcome by engineering alleles of the transposon or recombinase recognition sequence in which the frequency at which activated nucleotide sequences arise is reduced, such as with Ac(C1a) (Keller *et al.*, 1993). Alternatively, chemical or site-directed mutagenesis may be used to recover alleles of the necrosis-inducing genes which are less active and therefore result in less severe

levels of plant cell necrosis (Hammond-Kosack *et al.*, 1994).

In other systems, transposition or recombination may be inefficient resulting in too few activated nucleotide sequences leading to an insufficient level of plant cell necrosis. This may be overcome by constructing suitable promoter fusions to the transposase or recombinase gene in the plant gene (Swinburne *et al.*, 1992) to increase the frequency of excision or recombination to efficient levels. The most suitable promoter might give rise predominantly to late small sectors of necrosis during organ development rather than early large sectors.

Many other variations are possible as mechanisms for activating the defence response and/or necrosis after transposon excision or recombination. A form of the *Cf-9* gene may be constructed so that it activates the defence response even in the absence of its ligand. For example, the Drosophila receptor sevenless (involved in eye development) can be mutated so that it is activated in the absence of its ligand (Basler *et al*, 1991). For example, high level expression of a disease resistance gene, or expression of a disease resistance gene in another species, may lead to activation of the defence response and/or necrosis even in the absence of an avirulence product. Bonneus, *et al* (1995). In an alternative, the original disease

resistance gene may be mutated so that it binds to a defined chemical such as an agrichemical and this chemical activates *Cf-9* to initiate the defence response and/or necrosis. Hence, genotypic variegation for excision activating the gene may occur, without initiation of the somatic necrotic reaction due to the defence response. The defence response would be initiated when the agrichemical is applied and recognised by the resistance gene triggering the same reaction as if the avirulence gene product were present.

Introducing the Nucleotide Sequence or Sequences which Contribute to Variegation for the Plant Defence Response and/or Necrosis into the Plant Genome

The inactivated nucleotide sequence, or combination of nucleotide sequences at least one of which is inactivated, codes for a substance or substances which when expressed in the plant activates the defence response and/or leads to plant cell necrosis resulting in broad spectrum pathogen resistance.

The nucleic acid may be in the form of a recombinant vector, for example a plasmid or agrobacterium binary vector (Van den Elzen et al., 1985). The nucleic acid may be under the control of an appropriate promoter and regulatory elements for

expression in a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology,* Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

When introducing a chosen gene or gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The

nucleic acid to be inserted may be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material may or may not occur according to different embodiments of the invention. In a preferred embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. Finally, as far as plants are concerned the target cell type should be such that cells can be regenerated into whole plants.

Plants transformed with a DNA segment containing pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO

9012096, US 4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg. bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones

and glyphosate (Herrera-Estrella *et al*, 1983; van den Elzen *et al*, 1985).

The present invention is particularly beneficial for use in crop and amenity plants. Examples of suitable plants include tobacco, potato, pepper, cucurbits, carrot, vegetable brassicas, lettuce, strawberry, oil seed brassicas, sugar beet, wheat, barley, maize, rice, soybeans, peas, sunflower, carnation, chrysanthemum, other ornamental plants, turf grass, poplar, eucalyptus and pine.

Still further details of embodiments of the present invention are described in the following non-limiting examples, with reference to the accompanying drawings. In the drawings:

Figure 1 schematically depicts the *Cf-9* gene, showing tagged alleles. X marks a probable promoter.

Figure 2 illustrates genetic acquired resistance to *C. fulvum* induced following necrotic sector formation caused by the excision of a Ds element from the *Cf-9* resistance gene in an Avr9 expressing tomato plant. The number of *C. fulvum* pustules per leaf is indicated, 14 days after inoculation.

Figure 3 illustrates genetic acquired resistance to *Phytophthora infestans* (late blight of tomato and potato). GAR+ and GAR- plants from *Cf-9*Ds*, mutant

lines M31 and M50 and Cf0 plants spray inoculated with 10,000 sporangiospores/mL. In panel A the appearance of leaves from the mutant 50 experiment 7 days after inoculation is shown. In panel B the rate of leaf abscission (in days after inoculation) in the various genotypes inoculated is given.

Figure 4 illustrates genetic acquired resistance to *Phytophthora infestans* (late blight of tomato and potato). GAR+ and GAR- plants from *Cf-9*Ds*, mutant lines M31 and M50 and Cf0 plants were spray inoculated with 100 sporangiospores/mL. In panel A the appearance of leaves from the mutant 50 (GAR+ - right-hand) experiment 7 days after inoculation is shown, compared with GAR- (left-hand). In panel B the rate of sporulating lesion formation on the various plant genotypes inoculated is given, with the mean number of sporulating lesions/leaflet given at 5, 7, 10, 13 and 16 days after inoculation.

Figure 5 shows genetic acquired resistance to *Oidium lycopersici* (powdery mildew disease). GAR+ and GAR- plants from *Cf-9*Ds*, mutant lines M31 and M50 and Cf0 plants were painted with equivalent numbers of spores. In panel A the appearance of leaves 14 days after inoculation is shown, GAR- on the left, GAR+ on the right. In B, the rate of chlorotic lesion (upper panel) and sporulating lesion (lower panel) formation on the various plant genotypes is given for Mutant 31:

mean number of lesions given at 7, 10, 14, 21, 24 and 30 days after inoculation. C shows equivalent results for Mutant 50.

Figure 6 shows the appearance of tomato fruits on GAR$^+$ (*sAc, Cf-9*Ds* - right-hand) and GAR$^-$ (*sAc, Cf-9*Ds, Avr-9* - left-hand) plants from mutant line M23 at 2, 3, 4, 5, 6 and 7 weeks after flower pollination. Dark green sectors formed on the GAR$^+$ but not GAR$^-$ fruits by 5 weeks. These dark green sectors were not visible on the red fruit.

Figure 7 shows levels of defence-related gene expression in GAR+ and GAR- plants from Cf-9*Ds mutant lines M23, M31 and M50 just prior to the pathogen inoculation experiments. Northern analysis shows in panel A the levels of a basic β-1,3 glucanase gene transcript and in panel B the levels of an anionic peroxidase gene transcript.

Figure 8 illustrates functional expression of the *Cf-9* gene under the control of its own promoter in tobacco and potato. In panel A is shown a tobacco leaf that has been injected with intercellular fluid (IF) either containing the Avr9 peptide or lacking the Avr9 peptide. Avr9+ IF was obtained from transgenic tobacco or a compatible *C. fulvum* - tomato interaction involving race 5. Avr9- IF was obtained from untransformed tobacco or a compatible *C. fulvum* - tomato interaction involving race 2,4,5,9. Grey

necrosis was visible 3-4 h after injection only in the leaf panels that had received the Avr+ IF. In panel B four separate potato leaves are shown that have each been injected with a single type of IF. Only the two leaves that received the Avr9+IF developed grey necrosis by 24 h.

Figure 9 shows development of the necrotic lethal phenotype in seedlings from the tobacco cross cv. Petite Havana 6201A (*35S:SP:Avr9*)homozygote x cos 34.1 (genomic *Cf-9*) heterozygote. A time course for the period 5-12 days after seed planting (dsp) is shown. 50% of the seedlings become chlorotic and die within 2 days of seed germination.

Figure 10 shows development of the necrotic lethal phenotype in seedlings from the *Arabidopsis* cross 6201B4 (*35S:SP:Avr9*)heterozygote x cos 138 (genomic *Cf-9*) heterozygote. Appearance of seedlings 19 days after the majority of seedlings had germinated. One seedling has died and another has necrotic cotyledons.

Figure 11 shows a single T-DNA construct systems to apply GAR to potato plants. The T-DNA contains a *Cf-9* gene sequence under the control of its own promoter which has been inactivated by an autonomous *Ac* element that is only capable of a low level of excision, the *Ac* (Cla) element (Keller et al. 1993; Schofield et al. 1994) and the 35S:SP:Avr9 transgene.

Figure 12 shows a photograph of three leaves, two of which are diseased with *C. fulvum* and one which is expressing GAR and is resistant to the same inoculum of *C. fulvum*.

Figure 13 illustrates how GAR$^+$ plants may be made by crossing stable lines (1) comprising a *Cf-9* gene, inactivated by insertion of a *Ds* transposon, and an *Avr-9* gene and (2) an *Ac* transposase gene, as described in Example 1.

Figure 14 illustrates basic simplified haploid crossing schemes to produce plants with increased disease resistance.

T:	transgenic line
P:	offspring of transgenic line
T_1/P_1:	line comprising in its genome at least one of each of the four genes, R, L,I or A
$T_{1,2}/P_{1,2}$	line comprising in its genome at least one of each of two of the four genes R, L, I or A
T_3/P_3:	line comprising in its genome at least one of each of the four genes R,L,I or A not present in $T_{1,2}$
$T_{3,4}/P_{3,4}$:	line comprising in its genome at least one of two of the four genes R, L, I or A not present in $T_{1,2}$
$T_{1,2,3}/P_{1,2,3}$:	line comprising in its genome at

least one of each of three of the four genes R,L,I or A

T_4/P_4 line comprising in its genome at least one of each of the four genes R,L,I or A not present in $T_{1,2,3}$

SEQ ID NO. 1 shows the genomic DNA sequence of the *Cf-9* gene. Features: Nucleic acid sequence - Translation start at nucleotide 898; translation stop at nucleotide 3487; polyadenylation signal (AATAAA) at nucleotide 3703-3708; polyadenylation site at nucleotide 3823; a 115 bp intron in the 3' non-coding sequence from nucleotide 3507/9 to nucleotide 3622/4. Predicted Protein Sequence - primary translation product 863 amino acids; signal peptide sequence amino acids 1-23; mature peptide amino acids 24-863.

SEQ ID NO. 2 shows Cf-9 protein amino acid sequence.

SEQ ID NO. 3 shows the sequence of one of the *Cf-9* cDNA clones. Translation initiates at the ATG at position +58.Cf-9 genomic sequence

SEQ ID NO. 4 shows the amino acid sequence and DNA sequence of the preferred form of the chimaeric *Avr9* gene used as described herein.

SEQ ID NO. 5 shows the genomic DNA sequence of the *Cf-2.1* gene. Features: Nucleic acid sequence - Translation start at nucleotide 1677; translation stop

at nucleotide 5012; no consensus polyadenylation signal (AATAAA) exists in the characterised sequence downstream of the translation stop. Predicted Protein Sequence - primary translation product 1112 amino acids; signal peptide sequence amino acids 1-26; mature peptide amino acids 27-1112.

SEQ ID NO. 6 shows *Cf-2* protein amino acid sequence, designated *Cf*-2.1.

SEQ ID NO. 7 shows the amino acid sequence encoded by the *Cf-2.2* gene. Amino acids which differ between the two *Cf-2* genes are underlined.

SEQ ID NO. 8 shows the sequence of an almost full length cDNA clone which corresponds to the *Cf2-2* gene.

SEQ ID NO. 9 shows the genomic DNA sequence of the *RPP5* gene. Anticipated introns are shown in non-capitalised letters. Features: Nucleic acid sequence - Translation start at nucleotide 966; translation stop at nucleotide 5512.

SEQ ID NO. 10 shows predicted *RPP5* protein amino acid sequence.

SEQ ID NO. 11 shows genomic DNA sequence of *Cf-4*. Features of this sequence include: translation start site at nucleotide 201, translation stop beginning at nucleotide 2619, consensus polyadenylation sequence beginning at nucleotide 2835, splice donor sequence in 3' untranslated sequence at 2641, splice acceptor sequence ending at nucleotide 2755, proposed site of

polyadenylation at nucleotide 2955.

SEQ ID NO. 12 shows the predicted *Cf-4* amino acid sequence. The predicted protein sequence is composed of a primary translation product of 806 amino acids, signal peptide sequence amino acids 1-23, mature peptide amino acids 24-806.

SEQ ID NO. 13 shows double-stranded nucleic acid and deduced amino acid sequence of a *ClaI/SalI* DNA fragment encoding the PR1a signal peptide sequence fused to a sequence proposed to encode the mature processed form of *C. fulvum* AVR4. Translation initiation codon at nucleotide 5, termination codon beginning at nucleotide 413. Amino acids 1-30 represent the signal peptide and amino acids 31-136 the mature AVR4 peptide.

EXAMPLE 1

GENETIC ACQUIRED RESISTANCE (GAR) USING Cf-9

(i) Establishing a stock from which gametes carrying a mutagenised Cf-9 gene may be obtained and identified

During experiments to isolate the *Cf-9* gene by transposon tagging, alleles of the *Cf-9* gene (*Cf-9*Ds) were isolated that had been inactivated by insertion of the transposon *Ds* (See International Patent Application No. PCT/GB94/02812 for further details). This inactivated *Cf-9*Ds gene did not give rise to a

constitutive and lethal activation of defence mechanisms in response to the constitutively expressed 35S:SP:Avr9 gene.

We have established the capacity to carry out transposon tagging in tomato using the maize transposon Activator (Ac) and its Dissociation (*Ds*) derivatives (Scofield *et al* 1992; Thomas *et al* 1993; Carroll *et al* 1993). The strategy is founded on the fact that these transposons preferentially transpose to linked sites. Various lines that carry Dss at positions are useful, including FT33 (Rommens *et al* 1992), carrying a *Ds* linked to *Cf*-9, and lines that carry a construct SLJ10512 (Scofield *et al* 1992) which contains (a) a beta-glucuronidase (GUS) gene (Jefferson *et al* 1987) to monitor T-DNA segregation and (b) stable Ac (sAc) that expresses transposase and can trans-activate a *Ds*, but which will not transpose (Scofield *et al* 1992).

The line FT33 did not carry a *Cf*-9 gene. We had to obtain recombinants that placed *Cf*-9 in cis with the T-DNA in FT33 in order to carry out linked targeted tagging. Two strategies were pursued simultaneously:

(a) FT33 was crossed to Cf9, a stock that carries the *Cf*-9 gene. The resulting F1 was then back crossed to Cf0 (a stock that carries no Cf- genes). Progeny that carry the FT33 T-DNA are kanamycin resistant. Kanamycin resistant progeny were tested for the

presence of *Cf-9*; 5 *C. fulvum* resistant individuals were obtained among 180. We alsogenerated progeny that were homozygous for *Cf-9* and carried that sAc T-DNA of SLJ10512. These were crossed to the recombinants in which *Cf-9* and FT33 were *in cis*. In the FT33 T-DNA, a transposable *Ds* element is cloned into a hygromycin resistance gene, preventing its function. The somatic transactivation of this *Ds* element, which only occurs in the presence of transposase gene expression, results in activation of the hygromycin resistance. Thus from crossing the recombinants between *Cf-9* and FT33, to the sAc-carrying *Cf-9* homozygotes, hygromycin resistant individuals could be obtained which carry sAc and FT33, and are likely to be homozygous for *Cf-9*. 140 individuals of this genotype were thus obtained.

(b) To accelerate obtaining individuals that carried sAc, FT33, and were *Cf-9* homozygotes, the FT33/*Cf-9* F1 was crossed to a line that was heterozygous for *Cf-9* and sAc. 25% of the resulting progeny carried both T-DNAs and were hygromycin resistant, and of those, slightly more than 50% were disease resistant because they carried at least one copy of the *Cf-9* gene. An RFLP marker was available, designated CP46, that enabled us to distinguish between homozygotes and heterozygotes for the *Cf-9* gene (Balint-kurti *et al* 1993). In this manner two individuals that were *Cf-9* homozygotes, and that

carried both the FT33 T-DNA and sAc, were obtained. These two individuals were multiplied by taking cuttings so that more crosses could be made onto this genotype.

(ii) Establishing a tomato stock that expresses functional mature AVR9 protein

A likely frequency for obtaining any desired mutation in a gene tagging experiment is less than 1 in 1000, and often less than 1 in 10,000 (Döring, 1989). To avoid screening many thousands of plants for mutations to disease sensitivity, we established a selection for such mutations based on expressing the fungal *Avr9* gene in plants.

The sequence of the 28 amino acids of the mature Avr9 protein is known (van Kan *et al* 1991). It is a secreted protein and can be extracted from intercellular fluid of leaves infected with *Avr9*-carrying races of *C. fulvum*. For secretion from plant cells, we designed oligonucleotides to assemble a gene that carried a 30 amino acid plant signal peptide, from the Prla gene (Cornelissen *et al* 1987) preceding the first amino acid of the mature Avr9 protein (see SEQ ID NO. 4). The preferred *Avr9* gene sequence depicted in SEQ ID NO. 4 shows a chimaeric gene engineered from the Pr-1a signal peptide sequence (Cornelissen *et al*, 1987) and the *Avr9* gene sequence (van Kan *et al*, 1991). This

reading frame was fused to the 355 promoter of cauliflower mosaic virus (Odell *et al* 1984), and the 3' terminator sequences of the octopine synthase gene (DeGreve *et al* 1983), and introduced into binary plasmid vectors for plant transformation, using techniques well known to those skilled in the art, and readily available plasmids (Jones *et al* 1992). We obtained transformed Cf0 tomato lines that expressed this gene.

(iii) Crossing AVR9 expressing stock with Cf-9 expressing stock

The transformed lines obtained in (ii) were crossed to plants that carried the *Cf-9* gene. When the resulting progeny were germinated, 50% exhibited a necrotic phenotype, that culminated in seedling death. This outcome was only observed in seedlings that contained the *Avr9* gene. When the same transformants were crossed to Cf0 plants, the resulting progeny were all fully viable.

From selfing the primary transformants, individuals were identified that were homozygous for the *Avr9* transgene. When *Avr9* homozygotes were crossed to *Cf-9*, all progeny died. This system thus provides a powerful selection for individuals that carry mutations in the *Cf-9* gene.

(iv) Tagging and inactivating Cf-9

Individuals that were homozygous for the *Avr9* gene (section (iv)) were used as male parents to pollinate individuals that were homozygous for *Cf-9*, and carried both sAc and the *Ds* in the FT33 T-DNA (section (iiia) and (iiib)). Many thousands of progeny resulting from such a cross were germinated. Most died, but some survived.

DNA was obtained from survivors and subjected to Southern blot analysis using a *Ds* probe. It was observed that several independent mutations were correlated with insertions of the *Ds* into a BglII fragment of a consistent size. This suggested that several independent mutations were a consequence of insertion of the *Ds* into the same DNA fragment. Using primers to the *Ds* sequence, DNA adjacent to the *Ds* in transposed *Ds*-carrying mutant #18 was amplified using inverse PCR (Triglia *et al* 1988). This DNA was used as a probe to other mutants, and proved that in independent mutations, the *Ds* had inserted into the same 6.7 kb BglII fragment.

The *Ds* in FT33 contains a bacterial replicon and a chloramphenicol resistance gene as a bacterial selectable marker (Rommens *et al* 1992). This means that plant DNA carrying this transposed *Ds* can be digested with a restriction enzyme that does not cut within the *Ds* (such as BglII), the digestion products

can be recircularized, and then used to transform *E. coli*. Chloramphenicol resistant clones can be obtained that carry the *Ds* and adjacent plant DNA. This procedure was used to obtain a clone that carried 1.8 kb of plant DNA on the 3' side of the *Ds*, and 4.9 kb of plant DNA on the 5' side of the *Ds*.

Our present understanding of the *Cf-9* gene is depicted schematically in Figure 1. The *Cf-9* gene sequence and the deduced amino acid sequence are shown in the sequence listing.

A series of primers (F1, 2, 3, 4, 5, 6, 7, 12, 13, 10, 26, 27 and 25, indicated in Figure 1) was used to characterise a large number of independent mutations by PCR analysis in combination with primers based on the sequence of Ds. Therefore, these primers were used in polymerase chain reactions with primers based on the maize Ac/Ds transposon sequence, to characterise the locations of other mutations of *Cf-9* that were caused by transposon insertion. Eighteen independent insertions have been characterized and are located as shown. Mutants E, #55, #74 and #100 gave incomplete survival and showed a necrotic phenotype, and based on the available sequence information, they are 5' to the actual reading frame and might permit enough Cf9 protein expression to activate an incomplete defence response.

Using the sequence obtained of the gene,

oligonucleotide primers were designed that could be used in polymerase chain reactions in combination with primers based on the sequence of the *Ds* element, to characterize both the location and the orientation of other transposon insertions in the gene. These are shown on Figure 1. Based on the results of such experiments, the map positions of 17 other *Ds* insertions have been reliably assigned (as shown in Figure 1).

(v) Production of GAR plants

On backcrossing plants that carried the *Cf*-9*Ds and *35S:SP:Avr9* gene to tomato plants that carried an Ac transposase gene (sAc that lacked the GUS gene) in the homozygous state, but lacked *Cf*-9, one quarter of the resulting progeny carried sAc, *35S:SP:Avr9* and *Cf*-9*Ds (see Figure 13) plants showed somatic excision of *Ds* from the *Cf*-9*Ds gene, somatically restoring *Cf*-9 function, and giving rise to necrotic somatic sectors in which the defence response was activated. Phenotypically, these plants thus showed a variegation for a defence-related necrosis, in the same manner that plants challenged with necrotizing pathogens show somatic flecks of HR that are associated with the induction of SAR.

Necrotic sectors were visible on cotyledons, leaves, stems, petioles, sepals, and green fruits throughout plant development. Also, the necrotic

sectors formed in both the lower and upper epidermis, in all mesophyll layers and in the cells surrounding the vascular tissue. The size of the necrotic sector and the frequency of their formation was determined by both the position of the Ds element in the *Cf-9* sequence and the orientation of the *Ds*.

The plants that variegated for necrosis were tested to assess if they were more resistant to *C. fulvum* than their unvariegated siblings that either carried *Cf*-9*Ds or carried no *Cf*-9 gene. Plants from five independent *Cf-9*Ds* pedigrees were tested in which the Ds had independently inserted into five different locations in the *Cf-9* gene. These five independent insertions were between Cf-9 amino acids, 7 and 8 (<M23), 28 and 29 (<M18), 47 and 48 (>M50), 56 and 57 (>M31) and 789 and 790 (>M30) The arrows (< or >). indicates the direction of transcription of the *Ds* element. F_1 plants that developed somatic necrotic sectors were more resistant to *C. fulvum* than sibling offspring that did not develop necrotic sectors. On the plants with necrotic sectors an average of 1-2 small pustules per leaf developed, 14 days after inoculation with 5 x 10^5 spores/ml. The plants lacking a *Cf* gene and the non variegating individuals all showed on average 38 large sporulating pustules per leaf. A example of this is shown in Figure 2.

Nine variegated *Cf*-9*Ds #20 plants, fifteen

variegated *Cf*-9*Ds #23 plants, eighteen variegated *Cf*-9*Ds #30 plants and twenty-eight variegated *Cf*-9*Ds #31 plants were tested, and compared to one hundred and ninety eight plants in total that did not variegate for necrosis. Plants were inoculated with *C. fulvum* (5 x 10^5 spores/ml) when they were four weeks old and carried 2 expanded leaves. A similar result was obtained when variegated *Cf-9*Ds* #50 plants and non-variegated plants were inoculated with *C. fulvum*. On 18 variegated GAR^+ #50 plants 1-3 pustules per leaf formed, whereas on 42 non-variegated GAR^- #50 plants over 35 pustules per leaf developed by 14 days after inoculation.

Sensitivity to the pathogen was measured by counting the number of sporulating pustules that were visible on each genotype 14 days and 21 days after inoculation. Samples were also taken for microscopic analysis. The results of the assay after 14 days are shown in Figure 2, and typical infections on each genotype after 21 days are shown in Figure 12.

Figure 2 shows a histogram in which the sensitivity of different individual tomato plants is expressed on the y axis as the number of sporulating pustules per leaf. The *Ds* carried a GUS gene. M20, M23, M30 and M31 show *C. fulvum* growth on plants resulting from crosses between *Cf*-9*Ds and sAc, and derive from *Cf*-9*Ds #20, *Cf*-9*Ds #23, *Cf*-9*Ds #30 and

Cf-9*Ds #31, respectively. These individuals segregate from the *Cf*-9*Ds and for sAc. Cf0 carries no R genes and M20, M23, M30 and M31 GUS- plants have lost by segregation both *Cf*-9*Ds and sAc and are thus disease sensitive sibs, providing a good control for disease symptoms in sensitive individuals. If plants receive *Ds* without sAc they may be GUS+ without expressing the variegation for necrosis which requires both *Cf*-9*Ds and sAc. As can be seen, the necrotic individuals (which all carry the *35S:Avr9* gene) show distinctly fewer pustules per leaf than their disease sensitive sibs.

Figure 2 shows that in these experiments, Cf0 plants (lacking the *Cf-9* gene) exhibited about 38 pustules per leaf and non-variegating individuals derived from *Cf*-9*Ds #20, *Cf*-9*Ds #23 or *Cf*-9*Ds #31 also showed about 38 pustules per leaf. The non-variegated individuals that carried *Cf*-9*Ds #30 showed about 17 pustules per leaf indicating some residual action of the tagged *Cf*-9 allele. However, variegated individuals that carried *Cf*-9*Ds #20, *Cf*-9*Ds #23, *Cf*-9*Ds #30 or *Cf*-9*Ds #31 showed 1-3 pustules per leaf. In total seventy variegated individuals were assessed. These results demonstrate a very significant level of disease control by this method.

Figure 12 shows three leaves. Leaf 1 and Leaf 2 are infected with *C. fulvum* which confers the white

fluffy appearance. Leaf 1 is CfO and Leaf 2 is a disease sensitive sib from *Cf*-9*Ds #23. Leaf 3 showing minimal sporulation is a necrotic individual (small sectors of necrosis are discernible) that carried *Cf*-9*Ds #23, sAc and *35S:Avr9*. Leaf 3 is therefore expressing GAR.

It is important to recognize that in this example regions of variegating plants that resist the *C. fulvum* pathogen do not contain a functional *Cf*-9 gene. Indeed all the cells that do carry a functional *Cf*-9 gene (whose function was restored somatically by transposon excision) are killed as they turn on the defence response after recognition of the endogenously expressed *Avr9* peptide. Thus, non resistant cells are being induced to resistance by necrosis being manifested in adjacent cells.

EXAMPLE 2

Pathogen resistance of variegated plants employing Cf-9

In addition to demonstrating that variegated plants produced in Example 1 have enhanced resistance to *C. fulvum*, we have established that the plants are also more resistant to three unrelated fungal pathogens, *Phytophthora infestans* (the causal agent of late blight disease of tomato and potato) and *Oidium lycopersici* (a powdery mildew) and *Colletotrichum largenarium* (which causes leaf and fruit spot).

For the *P. infestans* experiments, sibling backcross progeny from the mutatnt *Cf-9* Ds* lines M31 and M50 that were either variegating for necrosis or not and control plants lacking a *Cf-gene* (Cf0) were challenged by a spray application of sporangiosspores (10,000 or 100 spores/ml) of the highly virulent isolate DSSI (A1 mating type). After inoculation, the plants were kept in diffuse light conditions at a constant 100% RH and 16°C and a 12h photoperiod.

Seven days after application of the high spore dose the leaves of the unvariegated plants and those of the Cf0 plants were completely destroyed by the spread of *P.infestans* lesions which had abundant sporangiospores at their margins. In contrast, the variegated plants were infected with *P. infestans* but the lesions were 3-5 mm in diameter and non-sporulating (Figure 3 A,B). An additional 5-6 days were required before the entire green leaf tissue of the variegated plants was destroyed and fungal sporulation commenced. At the lower spore dose, by 7 days after inoculation, an average of 8-10 large sporulating lesions were present on each leaf of the unvariegated and Cf0 plants whereas on the plants variegating for necrosis there were 1-2 small non-sporulating lesions per 10 leaves (Figure 4 A,B). A minimum of 18 plants were used for each genotype/spore.

For the *Oidium lycopersici* experiments the

identical plant genotypes were used. Each leaf was inoculated by brushing with an artist paintbrush the spores from a single 14 day old sporulating pustule over an entire upper surface. The inoculated plants were then kept under diffuse light conditions at 20°C during the 16 h photoperiod and at 18°C during the dark period. The RH was maintained at 70%.

By day 10 post inoculation 8-10 chlorotic lesions were evident on the leaves of the unvariegated and Cf0 plants and in 1-2 of these sporulation had commenced. By contrast on the variegated plants 1-2 smaller chlorotic non-sporulating lesions were present on each leaf (Figure 5). By day 14 post inoculation more than 20 sporulating lesions per leaf were present on the unvariegated plants and these were accompanied by severe chlorotic symptoms on the remainder of the leaf. On the variegated plants 2-4 small sporulating lesions were present per leaf (Figure 5A). An additional 7-10 days were required before a similar level of sporulation and chlorosis formed on the variegated leaves to that found on the unvariegated and Cf0 leaves at day 14 post-inoculation. (16 plants each).

EXAMPLE 3

Variegation in fruit

Dark green sectors formed on green tomato fruits of GAR plants, 5 weeks after flower pollination (Figure

6). These sectors were not visible once the tomato fruit had turned red, which is encouraging for potential commercial exploitation. When mature red fruit taken from GAR^+ and GAR^- plants were injected with 100µl of spores of *Colletotrichum laginarium* (10^4 spores/ml) only the GAR^- fruit exhibited the typical soft rot disease symptoms seven days later. Repeated inoculations of the GAR^+ fruit failed to cause disease.

Collectively, the above results attest to a very significant level of disease control that can be achieved in the plants variegating for restoration of Cf-9 gene function whilst constitutively expressing the *Avr9* gene. The data also indicate that the disease control achievable by this method is potentially broad spectrum because the four fungal pathogens controlled have very dissimilar modes of parasitism: *C. fulvum* is a biotroph that does not form haustoria and grows exclusively in the extracellular spaces of the leaf mesophyll layers; *O. Lycopersici* is also a biotroph but colonises only the upper leaf epidermis and forms complex intracellular haustoria; *P. infestans* and *C.largenarium* are hemibiotroph that initially forms simple haustoria but later on kills host cells in both the epidermal and mesophyll layers.

Homozygous *Cf-9*Ds, 35S:SPAvr9* lines have been established for the tomato lines M31 and M50. The F_1

backcross progeny derived from crosses to a homozygous sAc source, may be assessed for their resistance to various pathogens, including:

Potato virus X, *Pseudomonas syringae* pv. *tomato*, Necrotrophic fungi - *Botrytis spp*, *Colletotrichum spp*, Nematodes - *Meloidogyne incognata*, Aphids - Green Peach Aphid, and fruit, pod, root or tuber attacking pathogens. Also, the effect of GAR on the establishedment of mycorrhizal associations may be tested.

The enhanced resistance exhibited in the plants variegating for necrosis has been termed Genetic Acquired Resistance (GAR). It is distinct from SAR because it is a heritable trait and is active throughout the entire plants life.

When the expression of several defence-related genes were compared in the GAR^- and GAR^+ plants, significantly higher levels of expression of each gene were found in the GAR^+ plants. Examples of this are shown in Figure 7 for *Cf-9*Ds* lines from M23, M31 and M50 pedigrees using a basic tomato β-1,3 glucanase probe and a tomato anionic peroxidase probe (pTAP 4.5).

The effectiveness of GAR in suppressing plant disease appears to be inversely related to sector size. The two independent *Cf-9*Ds* pedigrees that have the highest frequency of small necrotic sectors (lines M31

and M50) give the best GAR. This indicates that by carefully manipulating the frequency of somatic restoration of *Cf-9* function even higher levels of plant protection be developed.

Currently, there are two possible hypotheses to explain GAR. Either the initially activated host cells generate local and systemic signals whilst still alive, and the necrotic lesions are a by-product of the *Cf-9-Avr9* mediated responses. Alternatively, the actual death and necrotic reactions, the final response of the activated host cells, generates specific local and systemic signals in a manner analogous to SAR. Exactly how GAR works does not need to be known for the present invention to be operated. Provided the required genetic components are present, GAR plants have enhanced pathogen resistance compared with wild-type.

EXAMPLE 4

Expression of Cf-9 in Heterologous Plants Species and Induction of Cell Necrosis

We have shown that following the transfer of different genomic clones containing the *Cf-9* gene into tobacco and potato, these sequences render the transgenic plants responsive to *Avr9* elicitor (Figure 8).

Also when transgenic tobacco expression *Cf-9* is crossed to transgenic tobacco plants engineered to

express *Avr9* peptide constitutively, the F1 seedlings die within 2 days of seed germination (Figure 9).

When transgenic *Arabidopsis* expressing *Cf-9* is crossed to *Avr9* expressing transgenic *Arabidopsis* the F1 seedlings die 10 days after seed germination (Figure 10).

Thus we have shown that in a variety of species, genes required for activation of plant defence, mediated by the Cf-9 protein, are present and functional.

EXAMPLE 5

Genetic Acquired Resistance Using Cf-9 in Potato

To apply GAR to potato plants a single T-DNA construct systems is used.

The system is based around a single T-DNA construct (Figure 11) containing, a *Cf-9* gene sequence under the control of its own promoter which has been inactivated by an autonomous *Ac* element that is only capable of a low level of excision (the *Ac* (Cla) element (Keller et al. 1993), and the 355:SP:Avr9 transgene). The *Ac* element is inserted at various positions in the *Cf-9* sequence and in both orientations in order to determine the best configuration to produce a high frequency of small somatic sectors where Cf-9 function has been restored.

Placing the *Cf-9* sequence or other R gene

sequence under the control of a cell-type specific promoter may enhance the GAR phenotype. Potential target cellular sites include the epidermis and the vascular parenchyma cells.

EXAMPLE 6

Expression of Cf-4 in transgenic plants and demonstration of increased pathogen resistance

The *Cf-4* gene has been tested in transgenic plants in a number of ways: firstly by inoculation with a race of *C. fulvum* containing the corresponding avirulence gene *Avr4* to test if that race gives an incompatible response on the transgenic plant; secondly by injecting leaves of a transformed plant with intercellular fluid isolated from a compatible interaction containing AVR4; thirdly, by delivering AVR4 in the form of recombinant potato virus X as described previously in studies of the Cf-9/AVR9 interaction (Hammond-Kosack *et al.*, 1995).

The DNA sequence of the *C. fulvum* gene encoding AVR4 has been reported and the amino acid sequence of the mature processed polypeptide (Joosten *et al.*, 1994). We amplified by PCR the *Avr4* gene from *C. fulvum* race 2,5 using primers to the published sequence and fused a sequence encoding the proposed mature polypeptide to a DNA sequence encoding the N-terminal

signal peptide of the tobacco PR1a protein. This would facilitate targeting of AVR4 to the intercellular space in transgenic plants where it is expressed. This chimeric gene (*SPAvr4*) was inserted into a cDNA copy of potato virus X, as a *ClaI/SalI* DNA fragment (SEQ ID NO. 13) as described previously (Hammond-Kosack *et al.*,1995) to generate PVX:*SPAvr4*. Infectious transcripts of the recombinant virus were generated by *in vitro* transcription. All nucleic acid manipulations were performed using standard techniques well known to those skilled in the art.

Tomato

Experiments were designed to test the recombinant virus in 3 week old tomato seedlings. In *Cf-4* containing plants inoculated cotyledons appeared desiccated and eventually abscised at 3 days post-inoculation (d.p.i.), in contrast to Cf0 controls which only showed signs of slight mechanical damage at the site of virus inoculation. Cf0 plants developed visible symptoms of virus infection at 7-10 d.p.i. comparable to symptoms observed with the wild type virus i.e. chlorotic mosaic symptoms. At 4-5 d.p.i. in plants containing *Cf-4* necrotic lesions were observed in the younger leaves, presumably due to systemic spread of the virus as described previously in similar experiments with PVX containing *Avr9* on *Cf-9* containing

plants (Hammond-Kosack *et al.*, 1995). Other features included necrotic sectors on petioles and the stem. The necrotic phenotype was seen to spread systemically and at 14 d.p.i. the majority of *Cf-4* containing seedlings had died. Cf0 control plants did not die but did show symptoms of chlorosis and vein-clearing.

These results confirm that *Cf-4* is functional in transgenic tomato plants, resulting in a necrotic defence response in the presence of elicitor AVR4.

Tobacco

Using binary vector cosmids comprising *Cf-4*, transgenic tobacco plants have also been produced (Fillatti *et al* .,1987; Horsch *et al.*, 1985) using techniques well known to those skilled in the art.

Transgenic tobacco containing cosmids comprising *Cf-4* were inoculated with PVX:*SPAvr4*. In most transformants necrotic lesions were observed at the site of virus inoculation 3-4 d.p.i. similar in appearance to lesions which appear in response to virus inoculation in some virus resistant varieties. In these individuals the necrosis was not strictly confined to local lesions which eventually coalesced and at 7-10 d.p.i. leaf necrosis was apparent over the entire region of virus inoculation. In several transformants the reaction to PVX:*SPAvr4* was more acute and the necrotic leaf sectors could be observed at 3-4

d.p.i. Neither of these phenotypes were observed in transgenic tobacco containing cosmids lacking *Cf-4* or in non-transformed control plants challenged with PVX:*SPAvr4*.

Functional expression of *Cf-4* in transgenic tobacco has thus also been shown, with activation of a necrotic defence response in the presence of elicitor AVR4.

Pathogen Resistance

Transgenic plants were propagated by cuttings so that *Cf-4* activity could be detected by inoculation with PVX:*SPAvr4* on 12 tomato transformants. Transgenic tomato plants containing *Cf-4* exhibited leaf necrosis on inoculated leaves 3-4 d.p.i. This necrosis eventually spread systemically as previously observed in *Cf-4* containing plants in the experiments described above. Transgenic plants exhibiting necrotic leaf sectors eventually died.

Cuttings of a number of transgenic plants obtained in the first round of transformation experiments were further assayed for *Cf-4* function by inoculation with *C. fulvum* race 5. In 5 transgenic plants tested, a positive correlation was observed between plants exhibiting PVX:*SPAvr4* dependent necrosis and resistance to the pathogen. In this experiment pathogen growth was observed on compatible control

plants (Cf0) but not on incompatible control plants (Cf2).

All documents mentioned in the text are incorporated herein by reference.

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SEQ ID NO. 1:

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AACATGCCAT GTCTGGACTC CTGCACTATC TTCCATCAAC AGGTCAATTC TCTCAACTCT     240

ATTGGTGGAA GGTAGACGGT ACAAATTGAA TTATATTAAA AGACAAGCTC ACCTGAGCAT     300

CACTGTTATA CAACAACAAC AAACTACGCT TCAGCCCCAA ACAATAGTGA CCCGAATCAT     360

ATATTGTCAC GAGTTTTTTT TAGAGTATGT TGCATATATT ATACTCAACT TAGGGTTTGT     420

CATTCTGATG CTTCGTACAA ATTTATTGAA TTTTCAACTT TAAAGGTTTA TGAACCAAAT     480

ATTACGCTTA CTATGATAGC GGTCTTTTTT GATTAATCAA ACTTATTGAA TTTTCAACTT     540

TAAAGGTTTT TCCCCGTTCT ATACACAAAC TAAGAAAAAT TTAAATTATA TAGTCTTTGG     600

ATGGTGACCT ATTTGGATGG TAACATTATT GGACCAAACT ATTGATAACG CGGACATTGT     660

TAGACCAAAC TGAGAAGGAC ATGTCTGGAC TCCTGCTCCG TCTTCCATCA GCAGGTCGAT     720

TCTTGTGGAA AATTAGCTCG AGGTGGCGCA CTATGTGAGG TAACTAGTAC TAAATTTTTC     780

TTTGCTTAAT TTGTGCTATA TATACCTCAT CTAAATTATT GAATAGTCAC ACAAAGCAAA     840

CATTTCTTGA TTTCTTCTCT ATCAACATAA CAAGTTTTGA TCATTTTTAG TGCAGAA        897

ATG GAT TGT GTA AAA CTT GTA TTC CTT ATG CTA TAT ACC TTT CTC TGT       945
Met Asp Cys Val Lys Leu Val Phe Leu Met Leu Tyr Thr Phe Leu Cys
-23          -20                 -15                 -10

CAA CTT GCT TTA TCC TCA TCC TTG CCT CAT TTG TGC CCC GAA GAT CAA       993
Gln Leu Ala Leu Ser Ser Ser Leu Pro His Leu Cys Pro Glu Asp Gln
         -5                   1                   5

GCT CTT TCT CTT CTA CAA TTC AAG AAC ATG TTT ACC ATT AAT CCT AAT      1041
Ala Leu Ser Leu Leu Gln Phe Lys Asn Met Phe Thr Ile Asn Pro Asn
 10                  15                  20                  25

GCT TCT GAT TAT TGT TAC GAC ATA AGA ACA TAC GTA GAC ATT CAG TCA      1089
Ala Ser Asp Tyr Cys Tyr Asp Ile Arg Thr Tyr Val Asp Ile Gln Ser
                 30                  35                  40

TAT CCA AGA ACT CTT TCT TGG AAC AAA AGC ACA AGT TGC TGC TCA TGG      1137
Tyr Pro Arg Thr Leu Ser Trp Asn Lys Ser Thr Ser Cys Cys Ser Trp
             45                  50                  55

GAT GGC GTT CAT TGT GAC GAG ACG ACA GGA CAA GTG ATT GCG CTT GAC      1185
Asp Gly Val His Cys Asp Glu Thr Thr Gly Gln Val Ile Ala Leu Asp
         60                  65                  70

CTC CGT TGC AGC CAA CTT CAA GGC AAG TTT CAT TCC AAT AGT AGC CTC      1233
Leu Arg Cys Ser Gln Leu Gln Gly Lys Phe His Ser Asn Ser Ser Leu
     75                  80                  85

TTT CAA CTC TCC AAT CTC AAA AGG CTT GAT TTG TCT TTT AAT AAT TTC      1281
Phe Gln Leu Ser Asn Leu Lys Arg Leu Asp Leu Ser Phe Asn Asn Phe
 90                  95                 100                 105

ACT GGA TCA CTC ATT TCA CCA AAA TTT GGT GAG TTT TCA AAT TTG ACG      1329
Thr Gly Ser Leu Ile Ser Pro Lys Phe Gly Glu Phe Ser Asn Leu Thr
                110                 115                 120
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CAT CTC GAT TTG TCG CAT TCT AGT TTT ACA GGT CTA ATT CCT TCT GAA      1377
His Leu Asp Leu Ser His Ser Ser Phe Thr Gly Leu Ile Pro Ser Glu
            125                 130                 135

ATC TGT CAC CTT TCT AAA CTA CAC GTT CTT CGT ATA TGT GAT CAA TAT      1425
Ile Cys His Leu Ser Lys Leu His Val Leu Arg Ile Cys Asp Gln Tyr
        140                 145                 150

GGG CTT AGT CTT GTA CCT TAC AAT TTT GAA CTG CTC CTT AAG AAC TTG      1473
Gly Leu Ser Leu Val Pro Tyr Asn Phe Glu Leu Leu Leu Lys Asn Leu
    155                 160                 165

ACC CAA TTA AGA GAG CTC AAC CTT GAA TCT GTA AAC ATC TCT TCC ACT      1521
Thr Gln Leu Arg Glu Leu Asn Leu Glu Ser Val Asn Ile Ser Ser Thr
170                 175                 180                 185

ATT CCT TCA AAT TTC TCT TCT CAT TTA ACA ACT CTA CAA CTT TCA GGC      1569
Ile Pro Ser Asn Phe Ser Ser His Leu Thr Thr Leu Gln Leu Ser Gly
                190                 195                 200

ACA GAG TTA CAT GGG ATA TTG CCC GAA AGA GTT TTT CAC CTT TCC AAC      1617
Thr Glu Leu His Gly Ile Leu Pro Glu Arg Val Phe His Leu Ser Asn
            205                 210                 215

TTA CAA TCC CTT CAT TTA TCA GTC AAT CCC CAG CTC ACG GTT AGG TTT      1665
Leu Gln Ser Leu His Leu Ser Val Asn Pro Gln Leu Thr Val Arg Phe
        220                 225                 230

CCC ACA ACC AAA TGG AAT AGC AGT GCA TCA CTC ATG ACG TTA TAC GTC      1713
Pro Thr Thr Lys Trp Asn Ser Ser Ala Ser Leu Met Thr Leu Tyr Val
    235                 240                 245

GAT AGT GTG AAT ATT GCT GAT AGG ATA CCT AAA TCA TTT AGC CAT CTA      1761
Asp Ser Val Asn Ile Ala Asp Arg Ile Pro Lys Ser Phe Ser His Leu
250                 255                 260                 265

ACT TCA CTT CAT GAG TTG TAC ATG GGT CGT TGT AAT CTG TCA GGG CCT      1809
Thr Ser Leu His Glu Leu Tyr Met Gly Arg Cys Asn Leu Ser Gly Pro
                270                 275                 280

ATT CCT AAA CCT CTA TGG AAT CTC ACC AAC ATA GTG TTT TTG CAC CTT      1857
Ile Pro Lys Pro Leu Trp Asn Leu Thr Asn Ile Val Phe Leu His Leu
            285                 290                 295

GGT GAT AAC CAT CTT GAA GGA CCA ATT TCC CAT TTC ACG ATA TTT GAA      1905
Gly Asp Asn His Leu Glu Gly Pro Ile Ser His Phe Thr Ile Phe Glu
        300                 305                 310

AAG CTC AAG AGG TTA TCA CTT GTA AAT AAC AAC TTT GAT GGC GGA CTT      1953
Lys Leu Lys Arg Leu Ser Leu Val Asn Asn Asn Phe Asp Gly Gly Leu
    315                 320                 325

GAG TTC TTA TCC TTT AAC ACC CAA CTT GAA CGG CTA GAT TTA TCA TCC      2001
Glu Phe Leu Ser Phe Asn Thr Gln Leu Glu Arg Leu Asp Leu Ser Ser
330                 335                 340                 345

AAT TCC CTA ACT GGT CCA ATT CCA TCC AAC ATA AGC GGA CTT CAA AAC      2049
Asn Ser Leu Thr Gly Pro Ile Pro Ser Asn Ile Ser Gly Leu Gln Asn
                350                 355                 360

CTA GAA TGT CTC TAC TTG TCA TCA AAC CAC TTG AAT GGG AGT ATA CCT      2097
Leu Glu Cys Leu Tyr Leu Ser Ser Asn His Leu Asn Gly Ser Ile Pro
            365                 370                 375

TCC TGG ATA TTC TCC CTT CCT TCA CTG GTT GAG TTA GAC TTG AGC AAT      2145
Ser Trp Ile Phe Ser Leu Pro Ser Leu Val Glu Leu Asp Leu Ser Asn
        380                 385                 390
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AAC ACT TTC AGT GGA AAA ATT CAA GAG TTC AAG TCC AAA ACA TTA AGT      2193
Asn Thr Phe Ser Gly Lys Ile Gln Glu Phe Lys Ser Lys Thr Leu Ser
    395                 400                 405

GCC GTT ACT CTA AAA CAA AAT AAG CTG AAA GGT CGT ATT CCG AAT TCA      2241
Ala Val Thr Leu Lys Gln Asn Lys Leu Lys Gly Arg Ile Pro Asn Ser
410                 415                 420                 425

CTC CTA AAC CAG AAG AAC CTA CAA TTA CTT CTC CTT TCA CAC AAT AAT      2289
Leu Leu Asn Gln Lys Asn Leu Gln Leu Leu Leu Leu Ser His Asn Asn
                430                 435                 440

ATC AGT GGA CAT ATT TCT TCA GCT ATC TGC AAT CTG AAA ACA TTG ATA      2337
Ile Ser Gly His Ile Ser Ser Ala Ile Cys Asn Leu Lys Thr Leu Ile
            445                 450                 455

TTG TTA GAC TTG GGA AGT AAT AAT TTG GAG GGA ACA ATC CCA CAA TGC      2385
Leu Leu Asp Leu Gly Ser Asn Asn Leu Glu Gly Thr Ile Pro Gln Cys
        460                 465                 470

GTG GTT GAG AGG AAC GAA TAC CTT TCG CAT TTG GAT TTG AGC AAA AAC      2433
Val Val Glu Arg Asn Glu Tyr Leu Ser His Leu Asp Leu Ser Lys Asn
    475                 480                 485

AGA CTT AGT GGG ACA ATC AAT ACA ACT TTT AGT GTT GGA AAC ATT TTA      2481
Arg Leu Ser Gly Thr Ile Asn Thr Thr Phe Ser Val Gly Asn Ile Leu
490                 495                 500                 505

AGG GTC ATT AGC TTG CAC GGG AAT AAG CTA ACG GGG AAA GTC CCA CGA      2529
Arg Val Ile Ser Leu His Gly Asn Lys Leu Thr Gly Lys Val Pro Arg
                510                 515                 520

TCT ATG ATC AAT TGC AAG TAT TTG ACA CTA CTT GAT CTA GGT AAC AAT      2577
Ser Met Ile Asn Cys Lys Tyr Leu Thr Leu Leu Asp Leu Gly Asn Asn
            525                 530                 535

ATG TTG AAT GAC ACA TTT CCA AAC TGG TTG GGA TAC CTA TTT CAA TTG      2625
Met Leu Asn Asp Thr Phe Pro Asn Trp Leu Gly Tyr Leu Phe Gln Leu
        540                 545                 550

AAG ATT TTA AGC TTG AGA TCA AAT AAG TTG CAT GGT CCC ATC AAA TCT      2673
Lys Ile Leu Ser Leu Arg Ser Asn Lys Leu His Gly Pro Ile Lys Ser
    555                 560                 565

TCA GGG AAT ACA AAC TTG TTT ATG GGT CTT CAA ATT CTT GAT CTA TCA      2721
Ser Gly Asn Thr Asn Leu Phe Met Gly Leu Gln Ile Leu Asp Leu Ser
570                 575                 580                 585

TCT AAT GGA TTT AGT GGG AAT TTA CCC GAA AGA ATT TTG GGG AAT TTG      2769
Ser Asn Gly Phe Ser Gly Asn Leu Pro Glu Arg Ile Leu Gly Asn Leu
                590                 595                 600

CAA ACC ATG AAG GAA ATT GAT GAG AGT ACA GGA TTC CCA GAG TAT ATT      2817
Gln Thr Met Lys Glu Ile Asp Glu Ser Thr Gly Phe Pro Glu Tyr Ile
            605                 610                 615

TCT GAT CCA TAT GAT ATT TAT TAC AAT TAT TTG ACG ACA ATT TCT ACA      2865
Ser Asp Pro Tyr Asp Ile Tyr Tyr Asn Tyr Leu Thr Thr Ile Ser Thr
        620                 625                 630

AAG GGA CAA GAT TAT GAT TCT GTT CGA ATT TTG GAT TCT AAC ATG ATT      2913
Lys Gly Gln Asp Tyr Asp Ser Val Arg Ile Leu Asp Ser Asn Met Ile
    635                 640                 645

ATC AAT CTC TCA AAG AAC AGA TTT GAA GGT CAT ATT CCA AGC ATT ATT      2961
Ile Asn Leu Ser Lys Asn Arg Phe Glu Gly His Ile Pro Ser Ile Ile
650                 655                 660                 665
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GGA GAT CTT GTT GGA CTT CGT ACG TTG AAC TTG TCT CAC AAT GTC TTG      3009
Gly Asp Leu Val Gly Leu Arg Thr Leu Asn Leu Ser His Asn Val Leu
                670                 675                 680

GAA GGT CAT ATA CCG GCA TCA TTT CAA AAT TTA TCA GTA CTC GAA TCT      3057
Glu Gly His Ile Pro Ala Ser Phe Gln Asn Leu Ser Val Leu Glu Ser
            685                 690                 695

TTG GAT CTC TCA TCT AAT AAA ATC AGC GGA GAA ATT CCG CAG CAG CTT      3105
Leu Asp Leu Ser Ser Asn Lys Ile Ser Gly Glu Ile Pro Gln Gln Leu
        700                 705                 710

GCA TCC CTC ACA TTC CTT GAA GTC TTA AAT CTC TCT CAC AAT CAT CTT      3153
Ala Ser Leu Thr Phe Leu Glu Val Leu Asn Leu Ser His Asn His Leu
    715                 720                 725

GTT GGA TGC ATC CCC AAA GGA AAA CAA TTT GAT TCG TTC GGG AAC ACT      3201
Val Gly Cys Ile Pro Lys Gly Lys Gln Phe Asp Ser Phe Gly Asn Thr
730                 735                 740                 745

TCG TAC CAA GGG AAT GAT GGG TTA CGC GGA TTT CCA CTC TCA AAA CTT      3249
Ser Tyr Gln Gly Asn Asp Gly Leu Arg Gly Phe Pro Leu Ser Lys Leu
                750                 755                 760

TGT GGT GGT GAA GAT CAA GTG ACA ACT CCA GCT GAG CTA GAT CAA GAA      3297
Cys Gly Gly Glu Asp Gln Val Thr Thr Pro Ala Glu Leu Asp Gln Glu
            765                 770                 775

GAG GAG GAA GAA GAT TCA CCA ATG ATC AGT TGG CAG GGG GTT CTC GTG      3345
Glu Glu Glu Glu Asp Ser Pro Met Ile Ser Trp Gln Gly Val Leu Val
        780                 785                 790

GGT TAC GGT TGT GGA CTT GTT ATT GGA CTG TCC GTA ATA TAC ATA ATG      3393
Gly Tyr Gly Cys Gly Leu Val Ile Gly Leu Ser Val Ile Tyr Ile Met
    795                 800                 805

TGG TCA ACT CAA TAT CCA GCA TGG TTT TCG AGG ATG GAT TTA AAG TTG      3441
Trp Ser Thr Gln Tyr Pro Ala Trp Phe Ser Arg Met Asp Leu Lys Leu
810                 815                 820                 825

GAA CAC ATA ATT ACT ACG AAA ATG AAA AAG CAC AAG AAA AGA TAT TAGTGAGTAG 3496
Glu His Ile Ile Thr Thr Lys Met Lys Lys His Lys Lys Arg Tyr
                830                 835                 840

CTATACCTCC AGGTATTCCA CTTGATCATT ATCTTTCAGA AGATTATTTT TTGTATATCG    3556

ATGAAATTAT CGACCTCCTT CATCCTCAAA GCTCTTAACT TTCACTCTTC ATTTTTGAAA    3616

ATTTCAGGAT TCAAAGATTT CCGAGTTCCC AGTTGCTTGG GATGCAGATA AAAGCCTTTT    3676

TATCTTTCAT AGTTTCTTAT CCTATGAATA AAGATTTTAT TTTCATTTGT CTATGGCACG    3736

TAGATATGTT CCGTCACTAA AAACATTGTA TTTCTCTCAA CTCTTTCGTC ACATGATATC    3796

AAAGAACACT TGACTTCAAT TAAGTTACTG TAGTCTGCTA TTTTAATTTT TTCCATTGAA    3856

ACACAACTGA CGTATCTTGA GAAAGAGACT ATGATCCCCC GGGCTGCAG                3905
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SEQ ID NO. 2:

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Met Asp Cys Val Lys Leu Val Phe Leu Met Leu Tyr Thr Phe Leu Cys
-23          -20                 -15                 -10

Gln Leu Ala Leu Ser Ser Ser Leu Pro His Leu Cys Pro Glu Asp Gln
         -5                  1                   5
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Ala Leu Ser Leu Leu Gln Phe Lys Asn Met Phe Thr Ile Asn Pro Asn
10 15 20 25

Ala Ser Asp Tyr Cys Tyr Asp Ile Arg Thr Tyr Val Asp Ile Gln Ser
30 35 40

Tyr Pro Arg Thr Leu Ser Trp Asn Lys Ser Thr Ser Cys Cys Ser Trp
45 50 55

Asp Gly Val His Cys Asp Glu Thr Thr Gly Gln Val Ile Ala Leu Asp
60 65 70

Leu Arg Cys Ser Gln Leu Gln Gly Lys Phe His Ser Asn Ser Ser Leu
75 80 85

Phe Gln Leu Ser Asn Leu Lys Arg Leu Asp Leu Ser Phe Asn Asn Phe
90 95 100 105

Thr Gly Ser Leu Ile Ser Pro Lys Phe Gly Glu Phe Ser Asn Leu Thr
110 115 120

His Leu Asp Leu Ser His Ser Ser Phe Thr Gly Leu Ile Pro Ser Glu
125 130 135

Ile Cys His Leu Ser Lys Leu His Val Leu Arg Ile Cys Asp Gln Tyr
140 145 150

Gly Leu Ser Leu Val Pro Tyr Asn Phe Glu Leu Leu Leu Lys Asn Leu
155 160 165

Thr Gln Leu Arg Glu Leu Asn Leu Glu Ser Val Asn Ile Ser Ser Thr
170 175 180 185

Ile Pro Ser Asn Phe Ser Ser His Leu Thr Thr Leu Gln Leu Ser Gly
190 195 200

Thr Glu Leu His Gly Ile Leu Pro Glu Arg Val Phe His Leu Ser Asn
205 210 215

Leu Gln Ser Leu His Leu Ser Val Asn Pro Gln Leu Thr Val Arg Phe
220 225 230

Pro Thr Thr Lys Trp Asn Ser Ser Ala Ser Leu Met Thr Leu Tyr Val
235 240 245

Asp Ser Val Asn Ile Ala Asp Arg Ile Pro Lys Ser Phe Ser His Leu
250 255 260 265

Thr Ser Leu His Glu Leu Tyr Met Gly Arg Cys Asn Leu Ser Gly Pro
270 275 280

Ile Pro Lys Pro Leu Trp Asn Leu Thr Asn Ile Val Phe Leu His Leu
285 290 295

Gly Asp Asn His Leu Glu Gly Pro Ile Ser His Phe Thr Ile Phe Glu
300 305 310

Lys Leu Lys Arg Leu Ser Leu Val Asn Asn Asn Phe Asp Gly Gly Leu
315 320 325

Glu Phe Leu Ser Phe Asn Thr Gln Leu Glu Arg Leu Asp Leu Ser Ser
330 335 340 345

Asn Ser Leu Thr Gly Pro Ile Pro Ser Asn Ile Ser Gly Leu Gln Asn
350 355 360

Leu Glu Cys Leu Tyr Leu Ser Ser Asn His Leu Asn Gly Ser Ile Pro
365 370 375

Ser Trp Ile Phe Ser Leu Pro Ser Leu Val Glu Leu Asp Leu Ser Asn
380 385 390

Asn Thr Phe Ser Gly Lys Ile Gln Glu Phe Lys Ser Lys Thr Leu Ser
395 400 405

Ala Val Thr Leu Lys Gln Asn Lys Leu Lys Gly Arg Ile Pro Asn Ser
410 415 420 425

Leu Leu Asn Gln Lys Asn Leu Gln Leu Leu Leu Leu Ser His Asn Asn
430 435 440

Ile Ser Gly His Ile Ser Ser Ala Ile Cys Asn Leu Lys Thr Leu Ile
445 450 455

Leu Leu Asp Leu Gly Ser Asn Asn Leu Glu Gly Thr Ile Pro Gln Cys
460 465 470

Val Val Glu Arg Asn Glu Tyr Leu Ser His Leu Asp Leu Ser Lys Asn
475 480 485

Arg Leu Ser Gly Thr Ile Asn Thr Thr Phe Ser Val Gly Asn Ile Leu
490 495 500 505

Arg Val Ile Ser Leu His Gly Asn Lys Leu Thr Gly Lys Val Pro Arg
510 515 520

Ser Met Ile Asn Cys Lys Tyr Leu Thr Leu Leu Asp Leu Gly Asn Asn
525 530 535

Met Leu Asn Asp Thr Phe Pro Asn Trp Leu Gly Tyr Leu Phe Gln Leu
540 545 550

Lys Ile Leu Ser Leu Arg Ser Asn Lys Leu His Gly Pro Ile Lys Ser
555 560 565

Ser Gly Asn Thr Asn Leu Phe Met Gly Leu Gln Ile Leu Asp Leu Ser
570 575 580 585

Ser Asn Gly Phe Ser Gly Asn Leu Pro Glu Arg Ile Leu Gly Asn Leu
590 595 600

Gln Thr Met Lys Glu Ile Asp Glu Ser Thr Gly Phe Pro Glu Tyr Ile
605 610 615

Ser Asp Pro Tyr Asp Ile Tyr Tyr Asn Tyr Leu Thr Thr Ile Ser Thr
620 625 630

Lys Gly Gln Asp Tyr Asp Ser Val Arg Ile Leu Asp Ser Asn Met Ile
635 640 645

Ile Asn Leu Ser Lys Asn Arg Phe Glu Gly His Ile Pro Ser Ile Ile
650 655 660 665

Gly Asp Leu Val Gly Leu Arg Thr Leu Asn Leu Ser His Asn Val Leu
670 675 680

Glu Gly His Ile Pro Ala Ser Phe Gln Asn Leu Ser Val Leu Glu Ser
685 690 695

Leu Asp Leu Ser Ser Asn Lys Ile Ser Gly Glu Ile Pro Gln Gln Leu
700 705 710

Ala Ser Leu Thr Phe Leu Glu Val Leu Asn Leu Ser His Asn His Leu
715 720 725

Val Gly Cys Ile Pro Lys Gly Lys Gln Phe Asp Ser Phe Gly Asn Thr
730 735 740 745

Ser Tyr Gln Gly Asn Asp Gly Leu Arg Gly Phe Pro Leu Ser Lys Leu
750 755 760

Cys Gly Gly Glu Asp Gln Val Thr Thr Pro Ala Glu Leu Asp Gln Glu
765 770 775

Glu Glu Glu Glu Asp Ser Pro Met Ile Ser Trp Gln Gly Val Leu Val
780 785 790

Gly Tyr Gly Cys Gly Leu Val Ile Gly Leu Ser Val Ile Tyr Ile Met
795 800 805

Trp Ser Thr Gln Tyr Pro Ala Trp Phe Ser Arg Met Asp Leu Lys Leu
810 815 820 825

Glu His Ile Ile Thr Thr Lys Met Lys Lys His Lys Lys Arg Tyr
830 835 840

SEQ ID NO. 3:

CATTTCTTGA TTTCTTCTCT ATCAACATAA CAAGTTTTGA TCATTTTTAG TGCAGAAATG 60

GATTGTGTAA AACTTGTATT CCTTATGCTA TATACCTTTC TCTGTCAACT TGCTTTATCC 120

TCATCCTTGC CTCATTTGTG CCCCGAAGAT CAAGCTCTTT CTCTTCTACA ATTCAAGAAC 180

ATGTTTACCA TTAATCCTAA TGCTTCTGAT TATTGTTACG ACATAAGAAC ATACGTAGAC 240

ATTCAGTCAT ATCCAAGAAC TCTTTCTTGG AACAAAAGCA CAAGTTGCTG CTCATGGGAT 300

GGCGTTCATT GTGACGAGAC GACAGGACAA GTGATTGCGC TTGACCTCCG TTGCAGCCAA 360

CTTCAAGGCA AGTTTCATTC CAATAGTAGC CTCTTTCAAC TCTCCAATCT CAAAAGGCTT 420

GATTTGTCTT TTAATAATTT CACTGGATCA CTCATTTCAC CAAAATTTGG TGAGTTTTCA 480

AATTTGACGC ATCTCGATTT GTCGCATTCT AGTTTTACAG GTCTAATTCC TTCTGAAATC 540

TGTCACCTTT CTAAACTACA CGTTCTTCGT ATATGTGATC AATATGGGCT TAGTCTTGTA 600

CCTTACAATT TTGAACTGCT CCTTAAGAAC TTGACCCAAT TAAGAGAGCT CAACCTTGAA 660

TCTGTAAACA TCTCTTCCAC TATTCCTTCA AATTTCTCTT CTCATTTAAC AACTCTACAA 720

CTTTCAGGCA CAGAGTTACA TGGGATATTG CCCGAAAGAG TTTTTCACCT TTCCAACTTA 780

CAATCCCTTC ATTTATCAGT CAATCCCCAG CTCACGGTTA GGTTTCCCAC AACCAAATGG 840

AATAGCAGTG CATCACTCAT GACGTTATAC GTCGATAGTG TGAATATTGC TGATAGGATA 900

CCTAAATCAT TTAGCCATCT AACTTCACTT CATGAGTTGT ACATGGGTCG TTGTAATCTG 960

TCAGGGCCTA TTCCTAAACC TCTATGGAAT CTCACCAACA TAGTGTTTTT GCACCTTGGT 1020

GATAACCATC TTGAAGGACC AATTTCCCAT TTCACGATAT TTGAAAAGCT CAAGAGGTTA 1080

TCACTTGTAA ATAACAACTT TGATGGCGGA CTTGAGTTCT TATCCTTTAA CACCCAACTT 1140

GAACGGCTAG ATTTATCATC CAATTCCCTA ACTGGTCCAA TTCCATCCAA CATAAGCGGA 1200

CTTCAAAACC TAGAATGTCT CTACTTGTCA TCAAACCACT TGAATGGGAG TATACCTTCC 1260

TGGATATTCT CCCTTCCTTC ACTGGTTGAG TTAGACTTGA GCAATAACAC TTTCAGTGGA 1320

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AAAGGTCGTA TTCCGAATTC ACTCCTAAAC CAGAAGAACC TACAATTACT TCTCCTTTCA 1440

CACAATAATA TCAGTGGACA TATTTCTTCA GCTATCTGCA ATCTGAAAAC ATTGATATTG 1500

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GAATACCTTT CGCATTTGGA TTTGAGCAAA AACAGACTTA GTGGGACAAT CAATACAACT 1620

TTTAGTGTTG GAAACATTTT AAGGGTCATT AGCTTGCACG GGAATAAGCT AACGGGGAAA 1680

GTCCCACGAT CTATGATCAA TTGCAAGTAT TTGACACTAC TTGATCTAGG TAACAATATG 1740

TTGAATGACA CATTTCCAAA CTGGTTGGGA TACCTATTTC AATTGAAGAT TTTAAGCTTG 1800

AGATCAAATA AGTTGCATGG TCCCATCAAA TCTTCAGGGA ATACAAACTT GTTTATGGGT 1860

CTTCAAATTC TTGATCTATC ATCTAATGGA TTTAGTGGGA ATTTACCCGA AAGAATTTTG 1920

GGGAATTTGC AAACCATGAA GGAAATTGAT GAGAGTACAG GATTCCCAGA GTATATTTCT 1980

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TTTGATTCGT TCGGGAACAC TTCGTACCAA GGGAATGATG GGTTACGCGG ATTTCCACTC 2400

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CTTGTTATTG GACTGTCCGT AATATACATA ATGTGGTCAA CTCAATATCC AGCATGGTTT 2580

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AGATATTAGT GAGTAGCTAT ACCTCCAGGA TTCAAAGATT TCCGAGTTCC CAGTTGCTTG 2700

GGATGCAGAT AAAAGCCTTT TTATCTTTCA TAGTTTCTTA TCCTATGAAT AAAGATTTTA 2760

TTTTCATTTG TCTATGGCAC GTAGATATGT TCCGTCACTA AAAACATTGT ATTTCTCTCA 2820

ACTCTTTCGT CACATGATAT CAAAGAACAC TTGACTTCAA TTAAGTTAAA AAAAAAAAAA 2880

SEQ ID NO. 4:

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ATG GGA TTT GTT CTC TTT TCA CAA TTG CCT TCA TTT CTT CTT GTC TCT        48
Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val Ser
 1               5                  10                  15

ACA CTT CTC TTA TTC CTA GTA ATA TCC CAC TCT TGC CGT GCC TAC TGT        96
Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala Tyr Cys
            20                  25                  30
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AAC AGT TCT TGT ACA AGA GCT TTT GAC TGT CTT GGA CAA TGT GGA AGA        144
Asn Ser Ser Cys Thr Arg Ala Phe Asp Cys Leu Gly Gln Cys Gly Arg
         35                  40                  45

TGC GAC TTT CAT AAG CTT CAA TGT GTA CAT TGA                        177
Cys Asp Phe His Lys Leu Gln Cys Val His
     50                  55
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SEQ ID NO. 5:

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   1  CTCGAGTTCG GAACCTAAAA GGTATAAAAT ATTAATAAAA ATTTTAAAAT
  51  GGTATATCAA TTTTTATATT AACCAAAACG TCAAAATCGC TGAAACAACA
 101  GCGATTTCCT TCACCGGAAA AAGCAAAATC GCTACTACTG CAGCGATTTT
 151  GCAAAATGTA ACTTTTTTTT AAAAAAATGC ATATTTTCTT ATAAGCTATA
 201  TATTTGAATT TCAAAAAAAA TATTTGAAAA TCAATAAAAT TTGTTTTTCC
 251  TACGATTTTC TTTTTAAAAT TCTTTTTTTG GAAAATCCCT ACCTAGGCAG
 301  CGATTTCCAT TTTTAATTTT TTTTAAATAA AAGGCAGCGA TTTTCGAAAA
 351  AAAAAATTTT AAAAAAAATT GAAAAAGTCG CTGCCTAGGT AGCGATTTGA
 401  ATTTTTTTAA AAAATGTTAT ATTTTGCAAA ATCGTTGCAG TAGCAACGAT
 451  TTTGCTTTTT TTGGAGGAAA TCGCTGTTGT TCCAGCGATT TTGCCGTTTT
 501  GGTTAATATA AAATTTTATA TAACGTTTTG AAATTTTTGT TAATATTTTA
 551  TAACTTTTAG GCTCCGGACT CAAGATTACT CCCTCTATCT TAGTTTATAA
 601  TGCATAGTCT GAATTTTGAA GAGCCAAATA GTTTAATTTT CGCCATAAAT
 651  TCAGACATGA AATCTTTAAA AAAGTTTAAA TAAAATTTGT ATATGTTGAA
 701  ACTACAGAAA AAGTATTATA ATTCACGATA ATTTATTCAC AAGCCATCGT
 751  CGGAGTGATC GCGAGTGAAG TGAAAGAATT GGAGTTTTTG ATATCCAGAA
 801  TCCATCTTGA GAGGTTGAGA TATCTTAATC TATCTCCAAT AAAAAAAAAC
 851  TATTAATATC CAATTTTCTT GAAGGCCATT ACCTATTCCG ACAAATTCCA
 901  CAAGATACTT CATCATATAA AAAAATAATC TCCGTGAAGA  AATTCTTTTA
 951  TTTGGAAAAT CGATTTTAGA GTCATTGCAA TTTAATTTTA TCAAAATATT
1001  TGAGCATGAA AAATTTGAAA TGGAGGTGTC ATAAAAATAA AATACCCTTT
1051  AAAACACGGC TTTATTGAGT TGACGATAGT TCAAGTAGGG AAAATAAATA
1101  ACTTATTAAT TGAATATAAA ACTTGCAAGA AAAAAGTGAT ATTCAAATTT
1151  AATTCTGACC ATTATCTCTT GATATTCTTT GCTCTTCATT TATTTGAATA
1201  TTCATTTTTC AAAAGTTCCA CGTCATAAGA CATCAAATAT CAAGTAGGTC
1251  CCATAAAAAT AAAATACCCT TCTCAACATG ACAAAGAAAG ATTGAAAAAT
1301  GACTAACATT TTCTCAAAGA CAAAAACAAA ACATGTGAGA GAAGACATTA
1351  CGAATCATCA TAATCTCTGA GACTGAGAAT TGTTAGATAT GGTCCACTAC
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1401 TGTAGAGATG AGAATTTTGA ACCAAATGTA TTATACACTA AGAGTGGTCA

1451 TGATCATTGT GTGATAACAA AACTATTTTG GCAACTTTGA CTCAGTCCTT

1501 GGCTAAATTA GACCTCTAAC ACAAAACAAT CCAAAAGTTG ACTTGAGAAT

1551 GACAACATTT TCTTCCCTGA TAGCAACCAA ATTAGCAAAT TTGGAAAAAA

1601 CGCGTGTCTT GTTGATCTTT AATTAGTATA AGTTACGTAC AATATCCTAT

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1801 AGAACCAGAA TAATTCCTTT TTGGCTTCAT GGATTCCAAG TTCTAATGCA

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1951 CATCCCTCCC TTCTCTTGAA AATCTTGATC TTAGCAAGAA CAATATCTAT

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2101 TAGCCAAGCT TCAGATCATC CGCATATTTC ACAATCAATT AAATGGATTT

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2201 TATCAACTTT CTTAGTGGTT CCATTCCTGC TTCAGTGGGG AATCTGAACA

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6201 TACTGAGATC AAGGATTTCT AAGTGCAGCC AATCTCTTCT CCAGTTCATC

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6301 AGTTACTGAG ATCAAAGAGC ATGAAAAAAG GCACTTCATA CTAATATGAT

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 101  DLSKNNIYGT IPPEIGNLTN LVYLDLNNNQ ISGTIPPQIG LLAKLQIIRI
 151  FHNQLNGFIP KEIGYLRSLT KLSLGINFLS GSIPASVGNL NNLSFLYLYN
 201  NQLSGSIPEE ISYLRSLTEL DLSDNALNGS IPASLGNMNN LSFLFLYGNQ
 251  LSGSIPEEIC YLRSLTYLDL SENALNGSIP ASLGNLNNLS FLFLYGNQLS
 301  GSIPEEIGYL RSLNVLGLSE NALNGSIPAS LGNLKNLSRL NLVNNQLSGS
 351  IPASLGNLNN LSMLYLYNNQ LSGSIPASLG NLNNLSMLYL YNNQLSGSIP
 401  ASLGNLNNLS RLYLYNNQLS GSIPEEIGYL SSLTYLDLSN NSINGFIPAS
 451  FGNMSNLAFL FLYENQLASS VPEEIGYLRS LNVLDLSENA LNGSIPASFG
 501  NLNNLSRLNL VNNQLSGSIP EEIGYLRSLN VLDLSENALN GSIPASFGNL
 551  NNLSRLNLVN NQLSGSIPEE IGYLRSLNDL GLSENALNGS IPASLGNLNN
 601  LSMLYLYNNQ LSGSIPEEIG YLSSLTYLSL GNNSLNGLIP ASFANMRNLQ
 651  ALILNDNNLI GEIPSSVCNL TSLEVLYMPR NNLKGKVPQC LGNISNLQVL
 701  SMSSNSFSGE LPSSISNLTS LQILDFGRNN LEGAIPQCFG NISSLEVFDM
 751  QNNKLSGTLP TNFSIGCSLI SLNLHGNELE DEIPRSLDNC KKLQVLDLGD
 801  NQLNDTFPMW LGTLPELRVL RLTSNKLHGP IRSSRAEIMF PDLRIIDLSR
 851  NAFSQDLPTS LFEHLKGMRT VDKTMEEPSY ESYYDDSVVV VTKGLELEIV
 901  RILSLYTVID LSSNKFEGHI PSVLGDLIAI RILNVSHNAL  QGYIPSSLGS
 951  LSILESLDLS FNQLSGEIPQ QLASLTFLEF LNLSHNYLQG CIPQGPQFRT
1001  FESNSYEGND GLRGYPVSKG CGKDPVSEKN YTVSALEDQE SNSEFFNDFW
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1101  GQRNYRRRNN HF*
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 101  DLSKNNIYGT IPPEIGNLTN LVYLDLNNNQ ISGTIPPQIG LLAKLQIIRI
 151  FHNQLNGFIP KEIGYLRSLT KLSLGINFLS GSIPASVGNL NNLSFLYLYN
 201  NQLSGSIPEE ISYLRSLTEL DLSDNALNGS IPASLGNMNN LSFLFLYGNQ
 251  LSGSIPEEIC YLRSLTYLDL SENALNGSIP ASLGNLNNLS FLFLYGNQLS
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301 GSIPEEIGYL RSLNVLGLSE NALNGSIPAS LGNLKNLSRL NLVNNQLSGS

351 IPASLGNLNN LSMLYLYNNQ LSGSIPASLG NLNNLSMLYL YNNQLSGSIP

401 ASLGNLNNLS RLYLYNNQLS GSIPEEIGYL SSLTYLDLSN NSINGFIPAS

451 FGNMSNLAFL FLYENQLASS VPEEIGYLRS LNVLDLSENA LNGSIPASFG

501 NLNNLSRLNL VNNQLSGSIP EEIGYLRSLN VLDLSENALN GSIPASFGNL

551 NNLSRLNLVN NQLSGSIPEE IGYLRSLNDL GLSENALNGS IPASLGNLNN

601 LSMLYLYNNQ LSGSIPEEIG YLSSLTYLSL GNNSLNGLIP ASFANMRNLQ

651 ALILNDNNLI GEIPSSVCNL TSLEVLYMPR NNLKGKVPQC LGNISNLQVL

701 SMSSNSFSGE LPSSISNLTS LQILDFGRNN LEGAIPQCFG NISSLEVFDM

751 QNNKLSGTLP TNFSIGCSLI SLNLHGNELE DEIPRSLDNC KKLQVLDLGD

801 NQLNDTFPMW LGTLPELRVL RLTSNKLHGP IRSSRAEIMF PDLRIIDLSR

851 NAFSQDLPTS LFEHLKGMRT VDKTMEEPSY ESYYDDSVVV VTKGLELEIV

901 RILSLYTVID LSSNKFEGHI PSVLGDLIAI RILNVSHNAL QGYIPSSLGS

951 LSILESLDLS FNQLSGEIPQ QLASLTFLEF LNLSHNYLQG CIPQGPQFRT

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1101 GQRNYRRRNN RF*

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3101  GCTTGAGAAG CTGTGGGATG GAACTCAGGT actaattttt ttagtgatca
3151  atttctaaac ataaaaacta aaaataaaaa tgtttaaaat gttcattaac
3201  gtgtgtgctc tcttttcccc tattttgttt tcagCCCCTT GGAAGTCTCA
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3351  GGAGACACTT CCTTCCTCGA TTCAGAATGC CATTAAACTG AGGGAGTTAA
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4051  TAGATTATCT CGAACAAGAT TGTTTCCGGA AGGGAGAAAT GAGATCGTGG
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5601  cgatcgtttg atatataatg cagatgacat cggggacatc tgaagaagat
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5801  gatgaacaaa tacctatctt gtatcctgtt tatggtaact gaagcatctt
5851  tatcattctg ttttgctctt ttttaggata acttgggatc gaccattatt
5901  ataaatttat aatgataatg acaaaacgat ttcataggtt ttgacttttg
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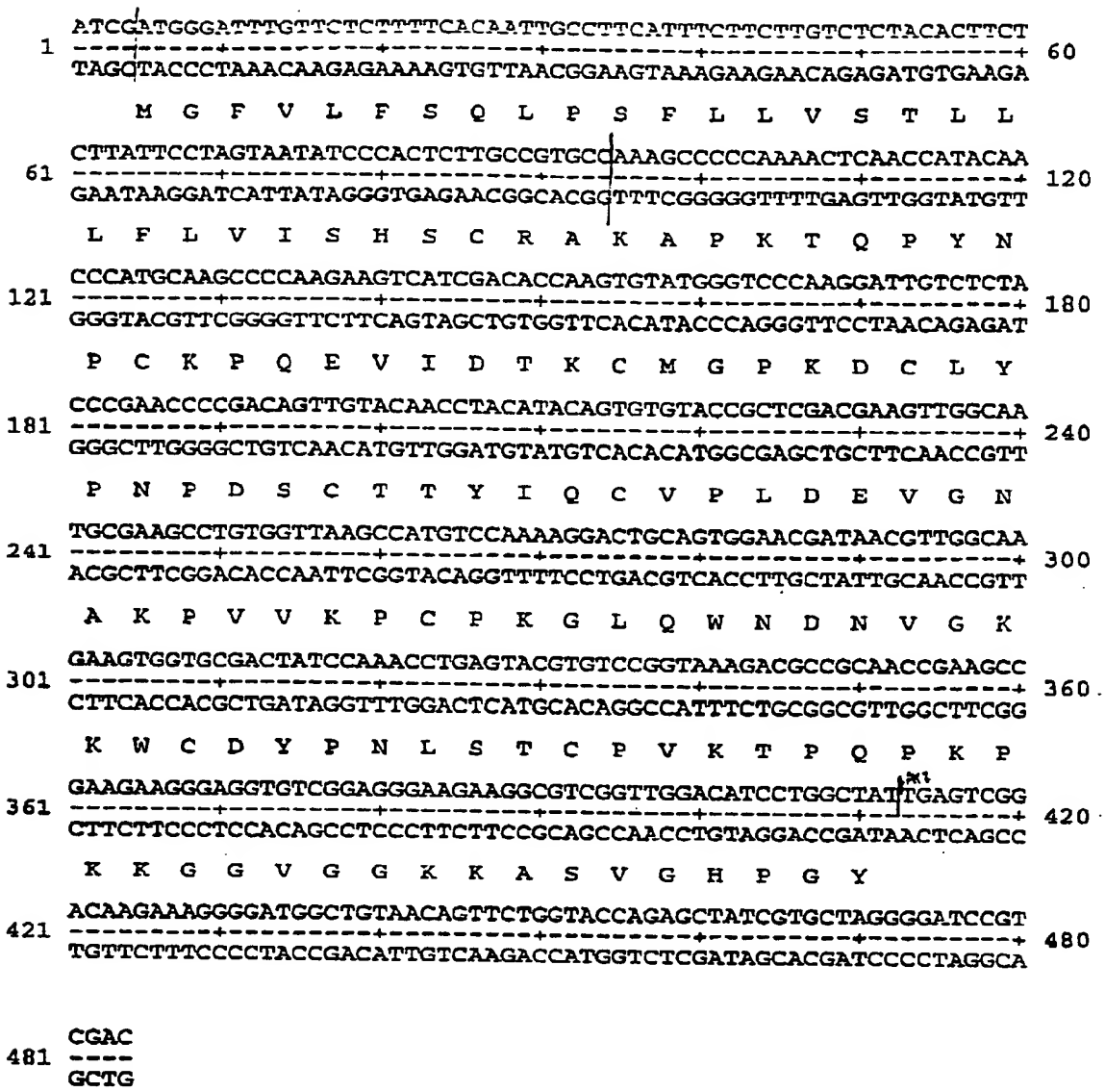


CLAIMS:

1. A method of providing increased pathogen resistance in a plant, or a part or propagule of a plant, by induction of variegation in which a gene is expressed or suppressed in cells resulting in the activation of a plant defence response, which comprises:

(i) inactivating a nucleotide sequence which contributes to a plant defence response or inactivating one or more nucleotide sequences forming a part of a combination of nucleotide sequences which contributes to a plant defence response;

(ii) introducing said nucleotide sequence or sequences into the genome of a plant; and

(iii) restoring said nucleotide sequence or sequences to a functional form in cells of the plant or a descendant thereof, or a part or propagule of the plant or descendant, to result in increased pathogen resistance.

2. A method of providing increased pathogen resistance in a plant, or a part or propagule thereof, by induction of variegation in which a gene is expressed or suppressed resulting in necrosis, which comprises:

(i) inactivating a nucleotide sequence which contributes to necrosis or inactivating one or more nucleotide sequences forming part of a combination of

nucleotide sequences which contributes to necrosis;
(ii) introducing said nucleotide sequence or sequences into the genome of a plant; and
(iii) restoring said inactivated nucleotide sequence or sequences to a functional form in cells of the plant or a descendant thereof, or a part or propagule of the plant or descendant, to result in necrosis.

3. A method according to claim 1 or claim 2 wherein said nucleotide sequence encodes or sequences encode a substance or a combination of substances which result in increased pathogen resistance.

4. A method according to any one of the preceding claims wherein said nucleotide sequence or sequences comprises a gene and activation of the plant defence response and/or necrosis due to the expression of said nucleotide sequence or sequences is not dependent on the expression of any other gene comprised in said nucleotide sequence or sequences.

5. A method according to any one of claims 1 to 3 wherein said nucleotide sequence or combination of nucleotide sequences comprises one or more genes and wherein activation of the plant defence response and/or necrosis due to the expression of said nucleotide sequence or sequences is conditional on the expression of one or more interacting genes.

6. A method according to claim 5 wherein said nucleotide sequences encodes or nucleotide sequences encode one or more substances which are or together are capable of inducing the plant defence response and/or necrosis, and at least one of said nucleotide sequences is inactivated in step (i).

7. A method according to claim 6 wherein said nucleotide sequence comprises a plant pathogen resistance gene (R) or a mutant, variant or derivative thereof, or a pathogen avirulence gene (*Avr*) or a mutant, variant or derivative thereof, or another R gene elicitor (E), or both (i) an R gene or a mutant, variant, or derivative thereof and (ii) a corresponding *Avr* gene, or a mutant, variant or derivative thereof, or another R gene elicitor (E).

8. A method according to claim 7 wherein said plant pathogen resistance gene (R) is a tomato *Cf-9* gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is *a Cladosporium fulvum Avr-9* gene or a mutant, variant, derivative or homologue thereof, or encodes another *Cf-9* elicitor.

9. A method according to claim 7 wherein said plant pathogen resistance gene (R) is a tomato *Cf-2* gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is *a Cladosporium fulvum Avr-2* gene

or a mutant, variant, derivative or homologue thereof, or encodes another *Cf-2* elictor; or wherein said plant pathogene resistance gene (R) is a tomato *Cf-4* gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is *a Cladosporium fulvum Avr-4* gene or a mutant, variant, derivative or homologue thereof, or encodes another *Cf-4* elictor; or wherein said plant pathogen resistance gene (R) is the tobacco N' gene or a mutant, variant, derivative or homologue thereof, and the avirulence gene is a suitable Tobacco Mosaic Virus coat protein, or a mutant, variant, derivative or homologue thereof or encodes another N' elicitor; or wherein said plant pathogen resistance gene (R) is the potato Rx gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is a suitable PVX coat protein or a mutant, variant, derivative or homologue thereof or another Rx elicitor; or wherein said plant pathogen resistance gene is another viral resistance gene and the avirulence gene encodes a corresponding viral coat protein or other elicitor of the viral resistance gene.

10. A method according to claim 5 wherein said nucleotide sequence encodes a Cauliflower Mosaic Virus gene VI protein, a bacterial harpin gene protein, an *Arabidopsis RPP5* gene protein, a ubiquitin conjugating enzyme, an RNase such as Barnase, a mutant, variant, derivative or homologue of any of these, or other toxic

polypeptide or peptide such as diphtheria toxin or a mutant, variant, derivative or homologue thereof.

11. A method according to claim 4 in which the plant defence response or necrosis is dependent on the expression from a nucleotide sequence leading to the reduction of expression of a gene that negatively regulates the plant defence response, resulting in the plant defence response and/or necrosis.

12. A method according to claim 4 in which the plant defence response or necrosis is dependent on the expression of an allele of a gene from a nucleotide sequence which activates the plant defence response in the absence of a ligand that is capable of interacting with the product of said gene, resulting in the plant defence response and/or necrosis.

13. A method according to claim 5 in which the plant defence response or necrosis is dependent on the expression of a mutant allele of a gene from a nucleotide sequence which is capable of activating the plant defence response and the expression of an enfeebled negative regulator of the defence response, leading to the plant defence response and/or necrosis.

14. A method according to any of the preceding claims wherein the inactivation of said nucleotide sequence or

of one or more of said nucleotide sequences is effected by the insertion therein of a transposable genetic element.

15. A method according to claim 14 wherein said transposable genetic element is a transposon or a nucleotide sequence bordered by specific nucleotide sequences that can be recognised by a site specific recombination system.

16. A method according to any of the preceding claims wherein said plant genome comprises at least one nucleotide sequence encoding a substance capable of restoring said inactivated nucleotide sequence or sequences to a functional form to result in increased pathogen resistance.

17. A method according to claim 16 which comprises restoring said inactivated nucleotide sequence or sequences to a functional form by excision or rearrangement of said transposable genetic element.

18. A method according to claim 17 wherein when said transposable element is a transposon, said plant genome comprises at least one nucleotide sequence coding for a corresponding transposon activation system to effect somatic excision of said transposon.

19. A method according to claim 18 wherein the genes encoding the transposon and transposase are derived from the Activator/Dissociation transposable element family (Ac/Ds) or from the Enhancer/Suppressor mutator transposon family (En/Spm).

20. A method according to claim 17 wherein when said inactive form of said nucleotide sequence or sequences is flanked by recombinase recognition sequences, said recombinase recognition sequences are acted on by a site specific recombination system which comprises a specific recombinase to result in recombination.

21. A transgenic plant, or descendant thereof, or part or propagule of the plant or descendant, obtainable using a method of any of the preceding claims with increased pathogen resistance compared with wild-type.

22. A plant, or a descendant thereof, or a part or propagule of the plant or descendant, or a derivative of any of these, which is phenotypically variegated, comprising a cell or clone expressing a first phenotype and other cells expressing a second phenotype comprising increased pathogen resistance compared with wild-type.

23. A plant, descendant, derivative, part or

propagule according to claim 22 wherein the first phenotype is necrosis and/or a plant defence response phenotype.

24. A plant, descendant, derivative, part or propagule according to claim 22 or claim 23 wherein the phenotypic variegation results from expression in cells with the first phenotype from a nucleotide sequence or sequences which contribute to such phenotype, said expression from said nucleotide sequence or sequences being inactivated in cells not having said first phenotype.

25. A plant, descendant, derivative, part or propagule according to claim 24 wherein said expression results from reactivation of a previously inactivated gene.

26. A plant, descendant, derivative, part or propagule according to claim 24 or claim 25 wherein said inactivation results from insertion of a transposable genetic element into said nucleotide sequence or one or more of said nucleotide sequences.

27. A plant, descendant, derivative, part or propagule according to any one of claims 24 to 26, wherein said nucleotide sequence or sequences comprises: a gene (R) which is a plant pathogen

resistance gene or a mutant, variant or derivative thereof; or a gene (L) which is a pathogen avirulence gene (*Avr*) or a mutant, variant or derivative thereof, or another elicitor or ligand gene the product of which can interact with the product of a R-gene; or both an R gene and an L gene.

28. A plant, descendant, derivative, part or propagule according to claim 27 wherein the R gene is a tomato *Cf-9* gene or a mutant, variant, derivative or homologue thereof and the L gene is *a Cladosporium fulvum Avr-9* gene or a mutant, variant, derivative or homologue thereof, or encodes another *Cf-9* elicitor.

29. A plant, descendant, derivative, part or propagule according to claim 27 wherein said R gene is:

(i) a pathogen resistance gene from tomato;

(ii) a pathogen resistance gene from tobacco;

(iii) a pathogen resistance gene from potato;

(iv) a pathogen resistance gene from *Arabidopsis*;

(v) a pathogen resistance gene from flax;

(vi) a nucleotide sequence encoding a CaMV gene VI protein;

(vii) a nucleotide sequence encoding a bacterial harpin gene protein;

(viii) a nucleotide sequence encoding a ubiquitin conjugating enzyme;

(ix) a nucleotide sequence encoding an RNase;

(x) a nucleotide sequence encoding a toxic peptide;

(xi) a mutant, variant, derivative or homologue of any of (i) to (x);

30. A plant, descendant, derivative, part or propagule according to claim 29 wherein said pathogen resistance gene from tomato is selected from *Cladosporium fulvum* resistance genes including *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*; said pathogen resistance gene from tobacco is N'; said pathogen resistance gene from potato is Nx; said pathogen resistance gene from *Arabidopsis* is *RPP5* or *RP52*; said pathogen resistance gene from flax is L6; said RNase is Barnase; or said toxic peptide is diphtheria toxin.

31. A plant, descendant, derivative, part or propagule according to claim 27 wherein said L gene is:

(i) a *Cladosporium fulvum* avirulence gene or another elicitor of a resistance gene for a *Cladosporium fulvum* avirulence gene;

(ii) a suitable TMV coat protein or another N' elicitor;

(iii) a suitable PVX coat protein or another Rx elicitor; or

(iv) a mutant, variant, derivative or homologue of any of (i) to (iii).

32. A plant, descendant, derivative, part or

propagule according to claim 31 wherein said *Cladosporium fulvum* avirulence gene is *Avr2*, *Avr4*, *Avr5* or *Avr9*.

33. A cell containing (i) nucleic acid encoding one or more than one nucleotide sequence which causes or contributes to the plant defence response and/or cell necrosis, at least one said nucleotide sequence being reversibly inactivated and (ii) nucleic acid encoding a molecule or molecules able to reverse the inactivation.

34. A cell according to claim 33 wherein the inactivation results from insertion of a transposable genetic element into one or more of said nucleotide sequences.

35. A cell according to claim 34 wherein said transposable genetic element is a transposon and said molecule or molecules provide a corresponding transposon activation system to effect excision of said transposon.

36. A cell according to any one of claims 33 to 35 wherein said nucleotide sequence or sequences comprises: a gene (R) which is a plant pathogen resistance gene or a mutant, variant or derivative thereof; or a gene (L) which is a pathogen avirulence gene (*Avr*) or a mutant, variant or derivative thereof,

or another elicitor or ligand gene the product of which can interact with the product of a R-gene; or both an R gene and an L gene.

37. A cell according to claim 36 wherein the R gene is a tomato *Cf*-9 gene or a mutant, variant, derivative or homologue thereof and the L gene is *a Cladosporium fulvum Avr-9* gene or a mutant, variant, derivative or homologue thereof, or encodes another *Cf-9* elicitor.

38. A cell according to claim 37 wherein said R gene is:

(i) a pathogen resistance gene from tomato;

(ii) a pathogen resistance gene from tobacco;

(iii) a pathogen resistance gene from potato;

(iv) a pathogen resistance gene from *Arabidopsis*;

(v) a pathogen resistance gene from flax;

(vi) a nucleotide sequence encoding a CaMV gene VI protein;

(vii) a nucleotide sequence encoding a bacterial harpin gene protein;

(viii) a nucleotide sequence encoding a ubiquitin conjugating enzyme;

(ix) a nucleotide sequence encoding an RNase;

(x) a nucleotide sequence encoding a toxic peptide;

(xi) a mutant, variant, derivative or homologue of any of (i) to (x);

39. A cell according to claim 38 wherein said pathogen resistance gene from tomato is selected from *Cladosporium fulvum* resistance genes including *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*; said pathogen resistance gene from tobacco is N'; said pathogen resistance gene from potato is Nx; said pathogen resistance gene from *Arabidopsis* is *RPP5* or *RP52*; said pathogen resistance gene from flax is L6; said RNase is Barnase; or said toxic peptide is diphtheria toxin.

40. A cell according to claim 36 wherein said L gene is:

(i) a *Cladosporium fulvum* avirulence gene or another elicitor of a resistance gene for a *Cladosporium fulvum* avirulence gene;

(ii) a suitable TMV coat protein or another N' elicitor;

(iii) a suitable PVX coat protein or another Rx elicitor; or

(iv) a mutant, variant, derivative or homologue of any of (i) to (iii).

41. A cell according to claim 40 wherein said *Cladosporium fulvum* avirulence gene is *Avr2*, *Avr4*, *Avr5* or *Avr9*.

42. A cell according to any one of claims 33 to 41 which is a microbial cell.

43. A cell according to any one of claims 33 to 41 which is a plant cell.

44. A plant or any part or propagule or derivative thereof comprising a cell according to claim 43.

45. A plant, part, propagule or derivative according to claim 44 which is variegated for cells wherein said nucleotide sequence is inactivated or activated.

46. A method of producing a cell according to any one of claims 33 to 45 comprising introduction of nucleic acid (i) and/or (ii) into the cell or an ancestor thereof.

47. A composition of matter comprising any of the following combinations of nucleotide sequences:
(i) a nucleotide sequence comprising R, a nucleotide sequence comprising I and a nucleotide sequence comprising A;
(ii) a nucleotide sequence comprising R, and a nucleotide sequence comprising I and A;
(iii) a nucleotide sequence comprising I, and a nucleotide sequence comprising A and R;
(iv) a nucleotide sequence comprising A, and a nucleotide sequence comprising R and I; and
(v) a nucleotide sequence comprising R, I and A;
wherein R encodes a substance whose presence in a plant

results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I.

48. A composition of matter comprising any of the following combinations of nucleotide sequences:

(i) a nucleotide sequence comprising R, a nucleotide sequence comprising L, a nucleotide sequence comprising I, and a nucleotide sequence comprising A;

(ii) a nucleotide sequence comprising R, a nucleotide sequence comprising L and I, and a nucleotide sequence comprising (A);

(iii) a nucleotide sequence comprising R, a nucleotide sequence comprising L and A, and a nucleotide sequence comprising I;

(iv) a nucleotide sequence comprising R, a nucleotide sequence comprising I and A, and a nucleotide sequence comprising L;

(v) a nucleotide sequence comprising L, a nucleotide sequence comprising I and R, and a nucleotide sequence comprising A;

(vi) a nucleotide sequence comprising L, a nucleotide sequence comprising A and R, and a nucleotide sequence comprising I;

(vii) a nucleotide sequence comprising I, a nucleotide sequence comprising L and R, and a nucleotide sequence comprising A;

(viii) a nucleotide sequence comprising R, and a nucleotide sequence comprising L, I and A;

(ix) a nucleotide sequence comprising L, and a nucleotide sequence comprising I, A and R;

(x) a nucleotide sequence comprising I, and a nucleotide sequence comprising A, R and L;

(xi) a nucleotide sequence comprising A and a nucleotide sequence comprising A, R and I; and

(xii) a nucleotide sequence comprising R, L, I and A;

wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I.

49. A composition of matter according to claim 47 or 48 which is one or more nucleic acid vectors.

50. A composition of matter according to any one of claims 47 to 49 wherein a cell contains any of said combinations of nucleotide sequences.

51. A plant, or a part, propagule, derivative or descendant thereof, comprising a cell according to the composition of claim 50.

52. A method of producing a plant, or a part,

propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, I and A, wherein R encodes a substance whose presence in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I, comprising crossing plant lines whose genomes comprise any of R, I, A and combinations thereof, to produce the plant or an ancestor thereof.

53. A method according to claim 52 wherein one or more of said plant lines contains nucleic acid comprising any of R, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof.

54. A method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, L, I and A, wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I, comprising crossing plant lines whose genomes

comprise any of R, L, I, A and combinations thereof, to produce the plant or an ancestor thereof.

55, A method according to claim 54 wherein one or more of said plant lines contains nucleic acid comprising any of R, L, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof.

56. A plant, or a part, propagule, derivative or descendant thereof, obtainable using a method according to any one of claims 52 to 55.

FIGURE 1

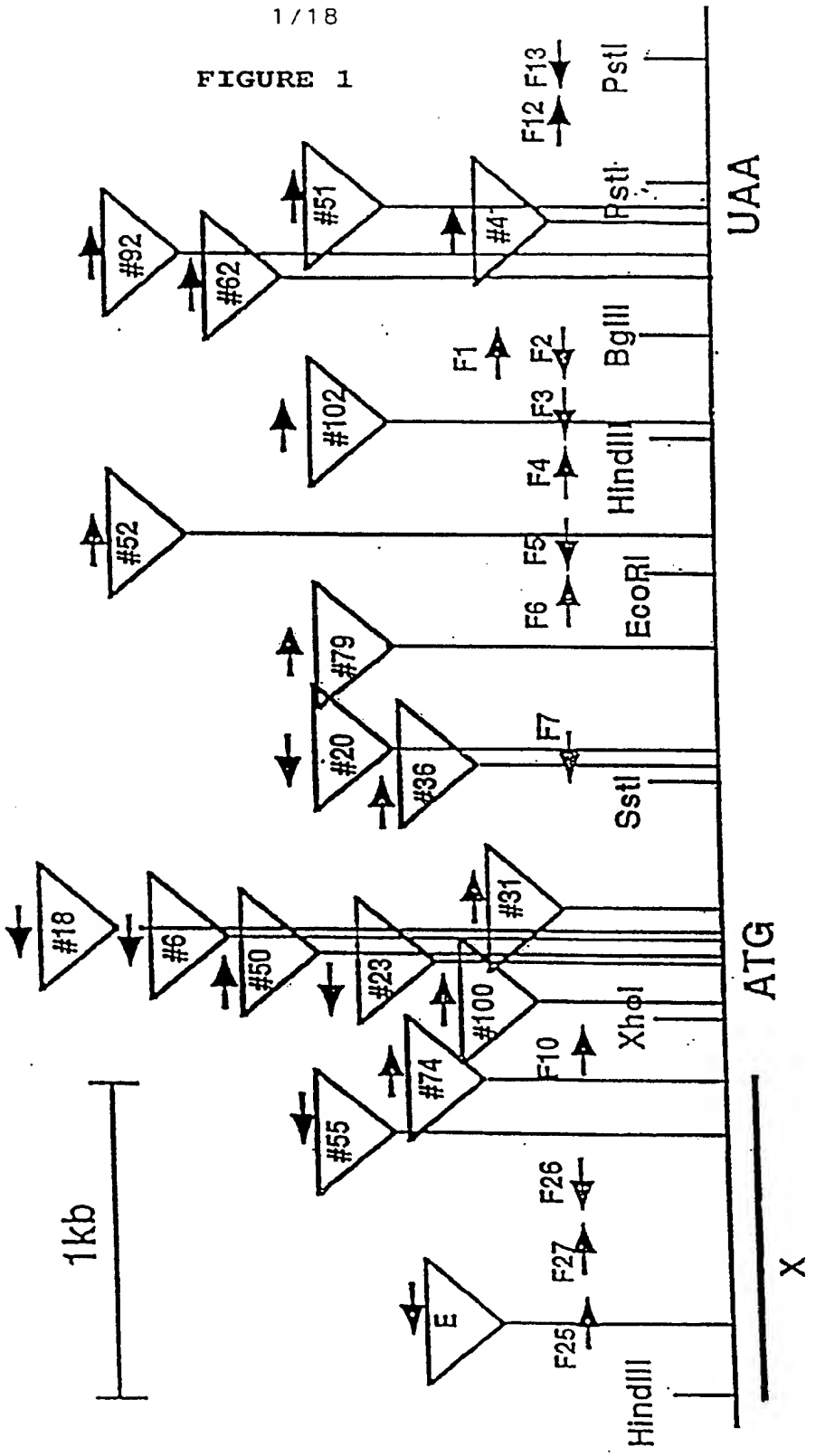


FIGURE 2

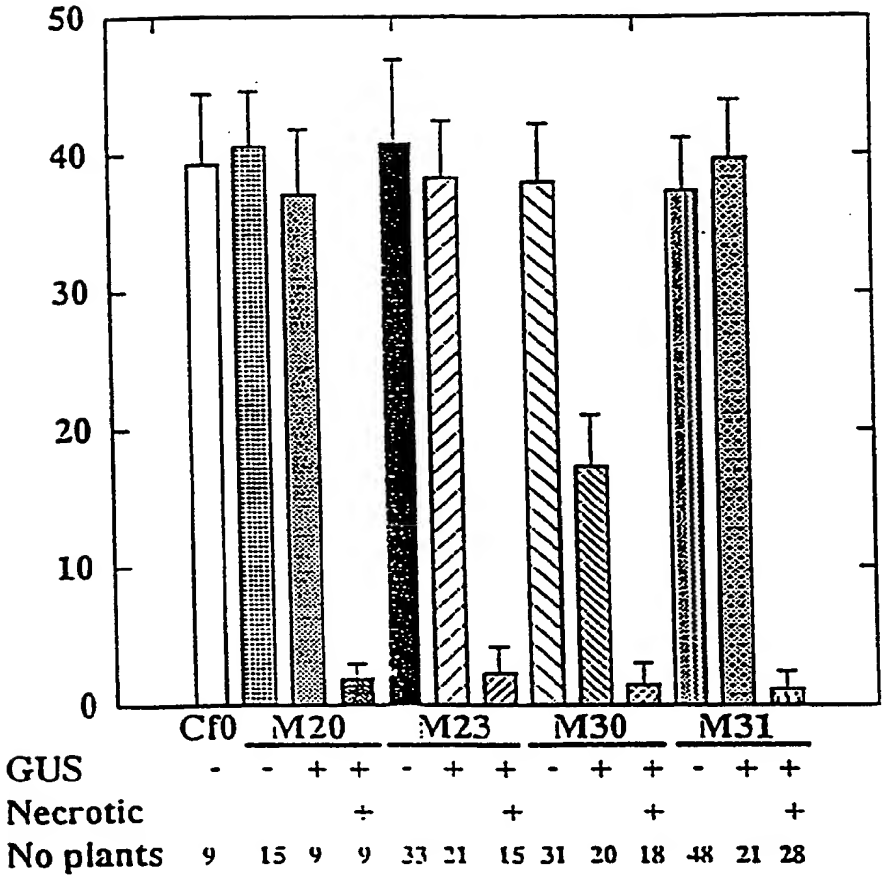


FIGURE 3A

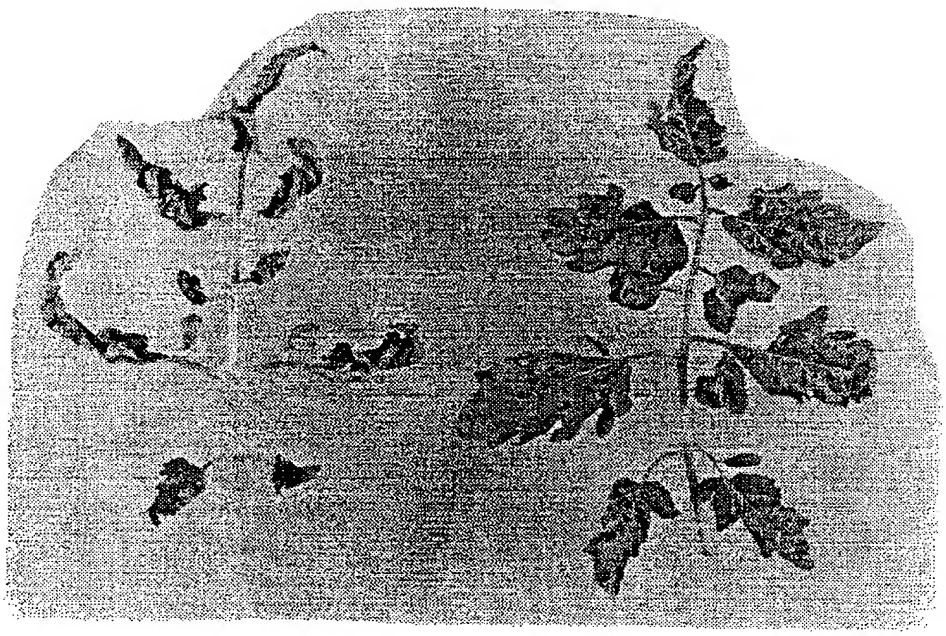
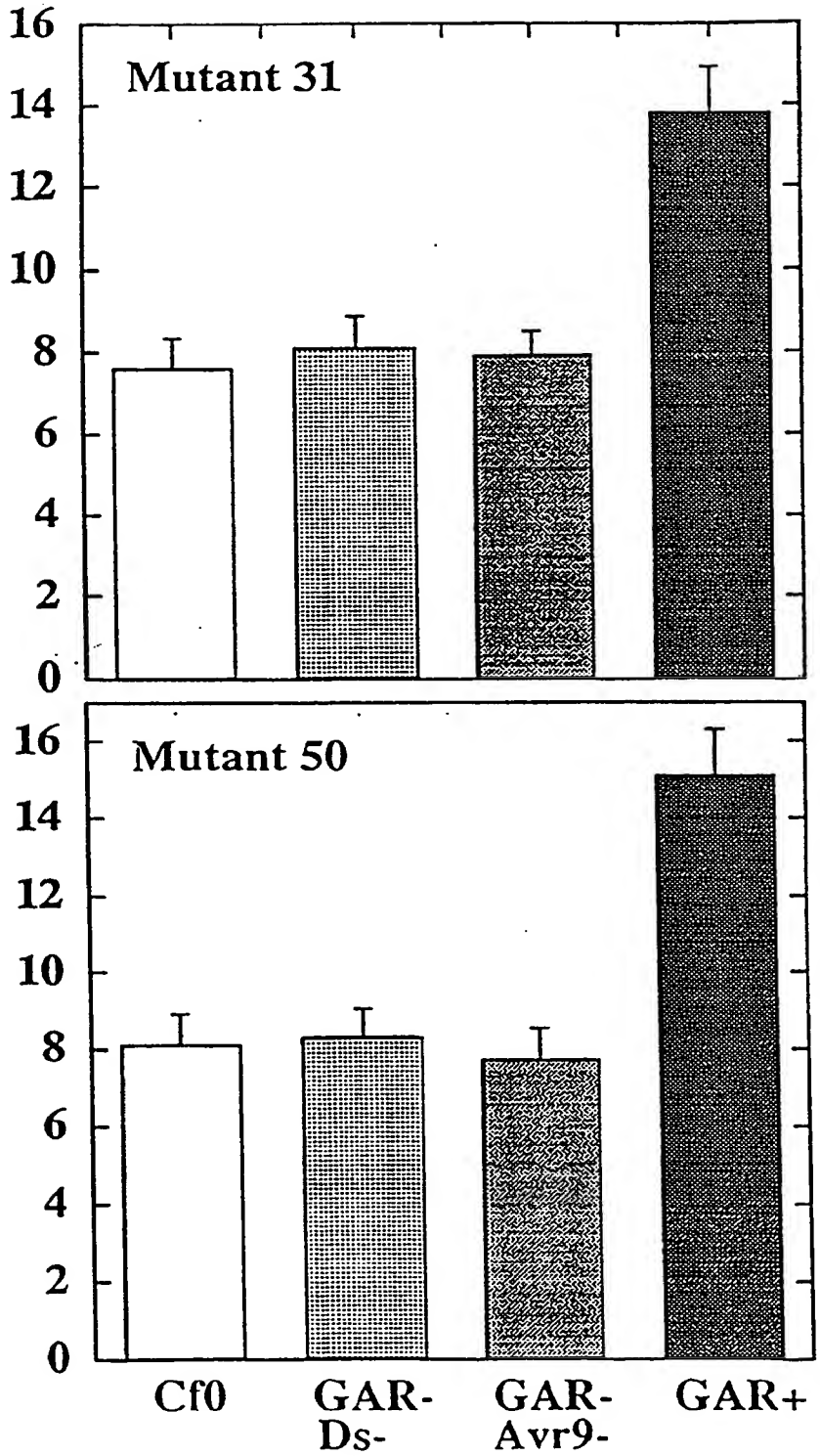


FIGURE 3B



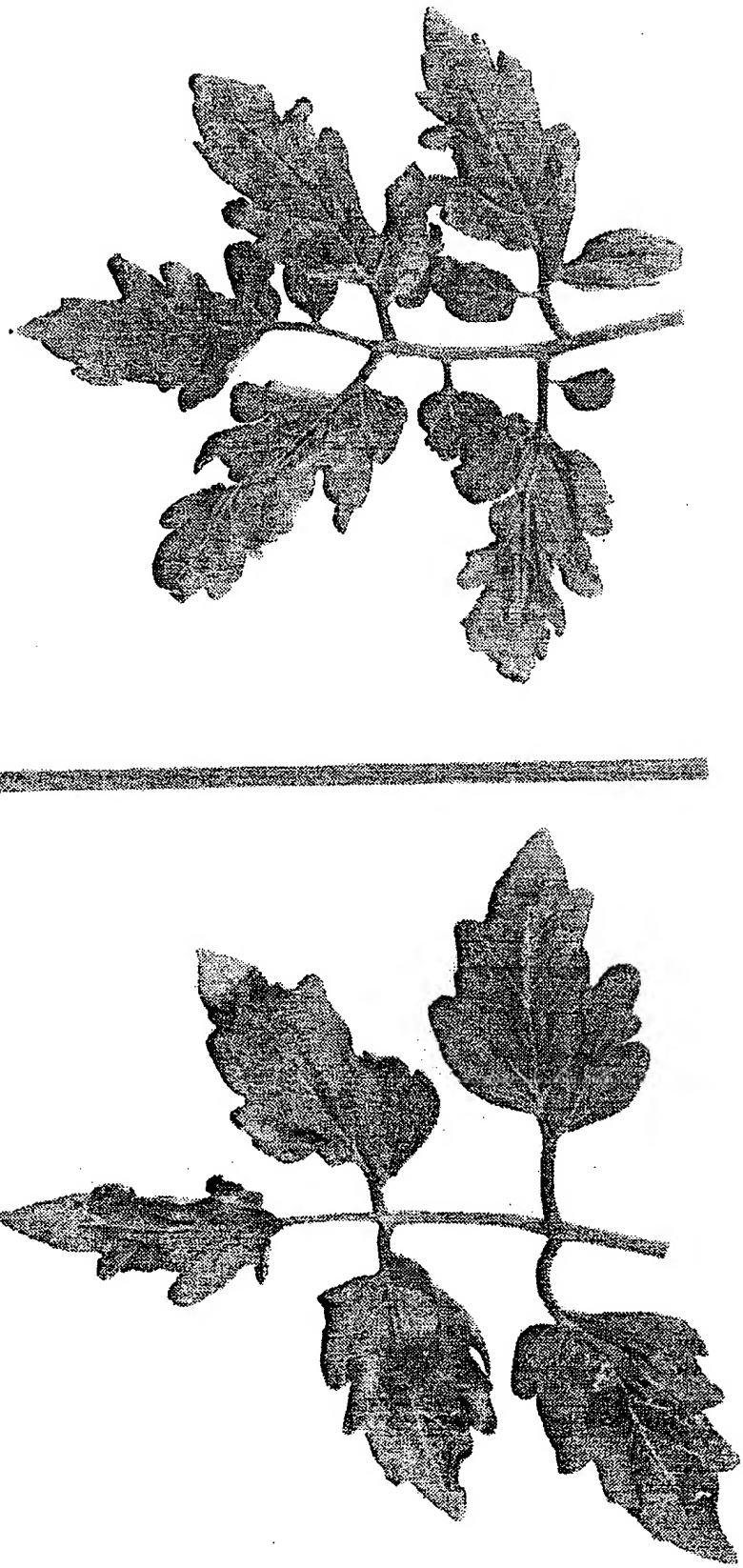
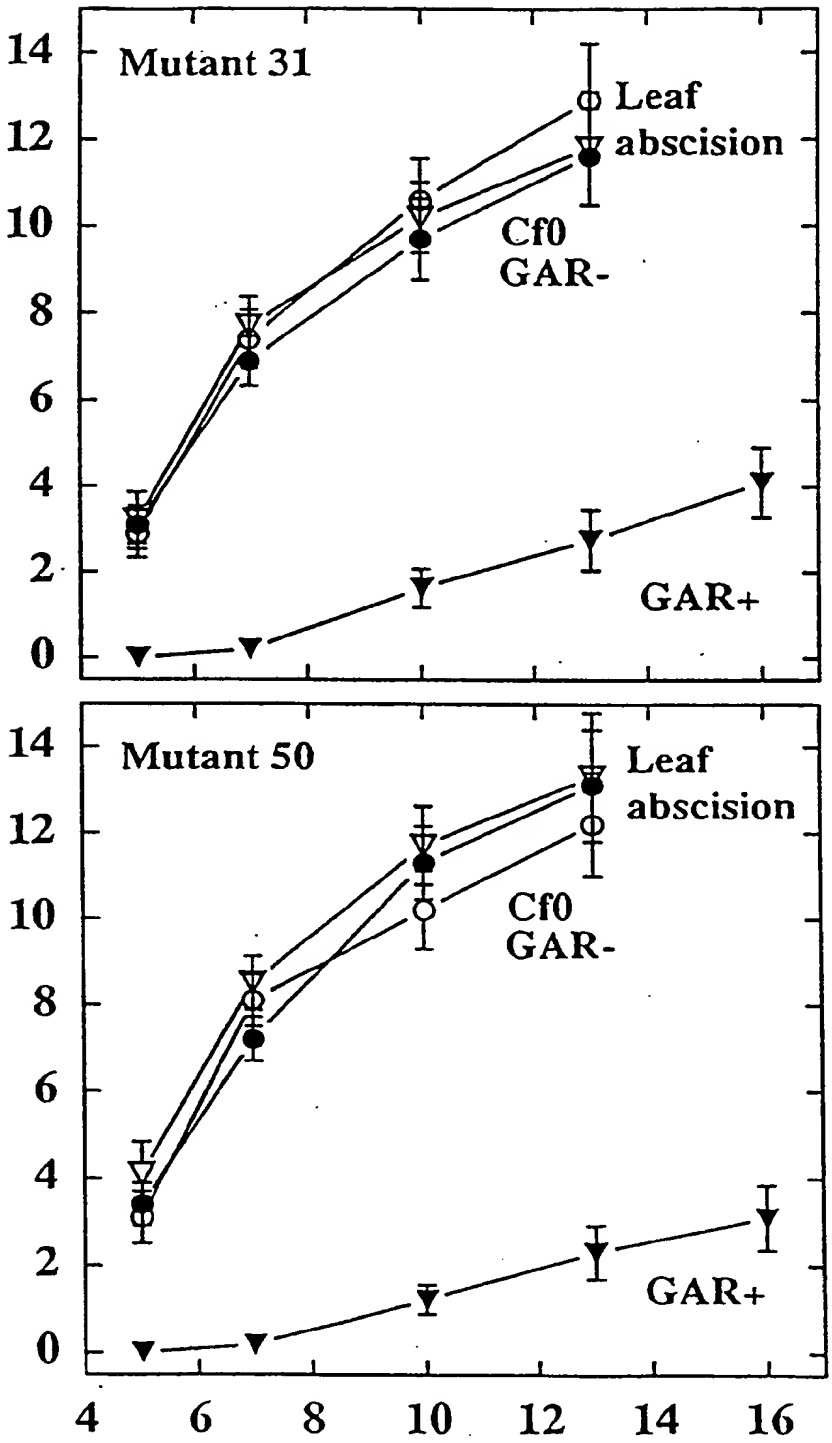


FIGURE 4A

FIGURE 4B



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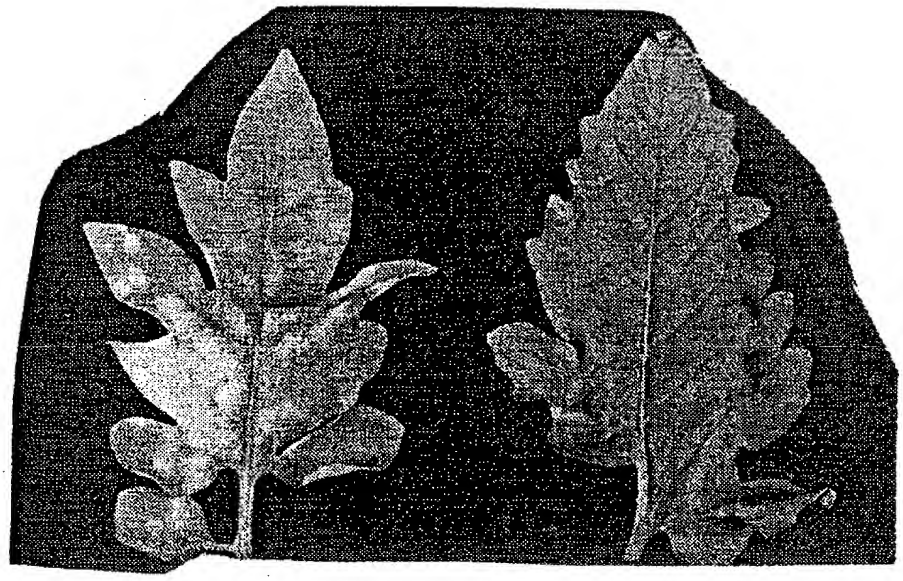
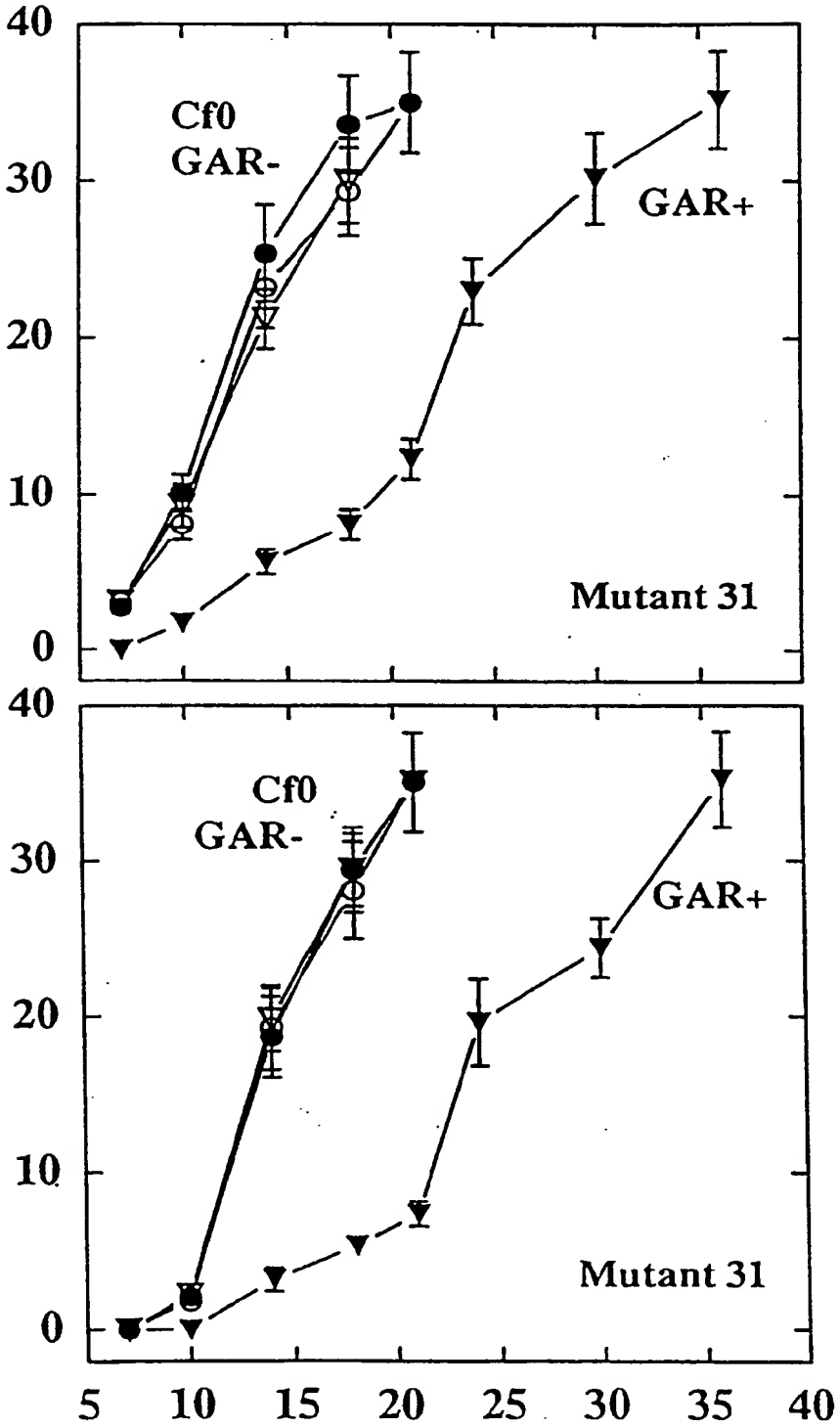


FIGURE 5B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30
20
10
0
Cf0
GAR-
GAR+
Mutant 31
40
30
20
10
0
Cf0
GAR-
GAR+
Mutant 31
5
10
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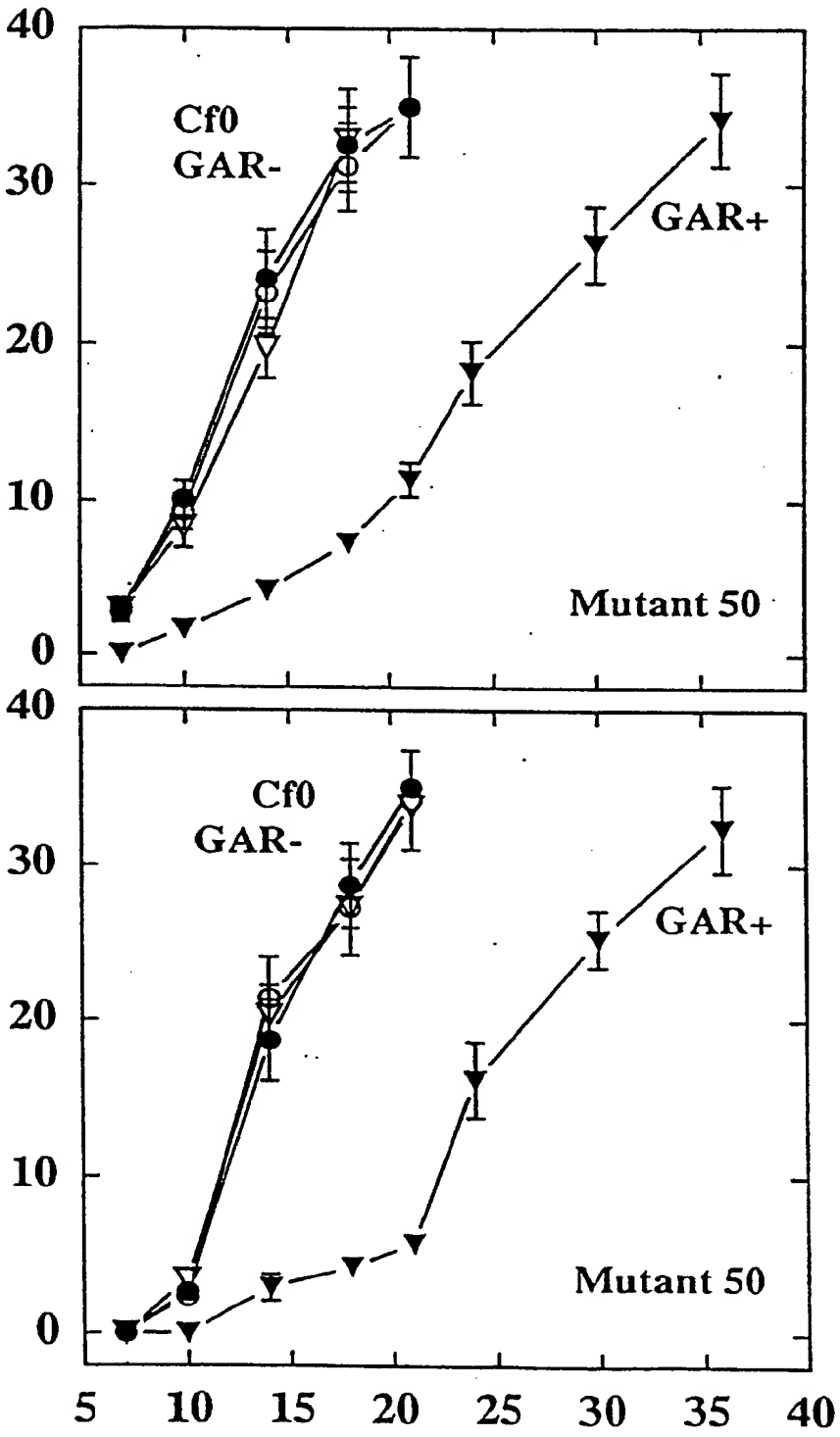
20
25
30
35
40

FIGURE 5C



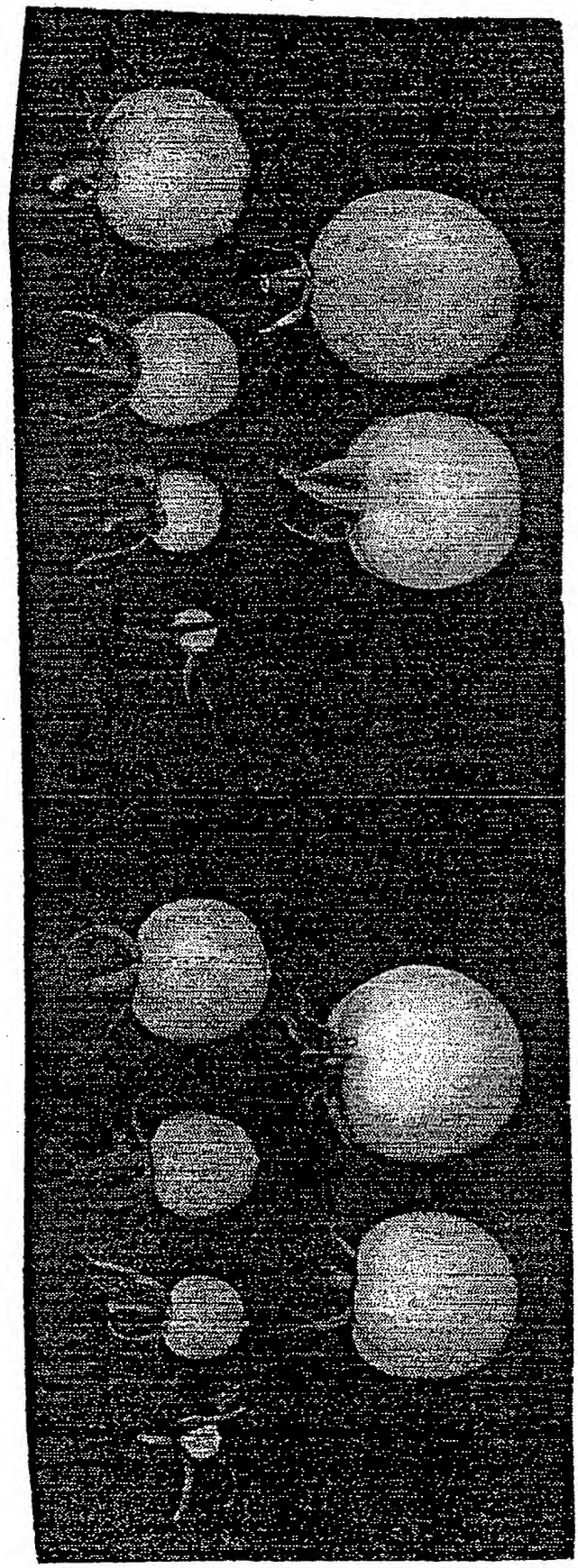
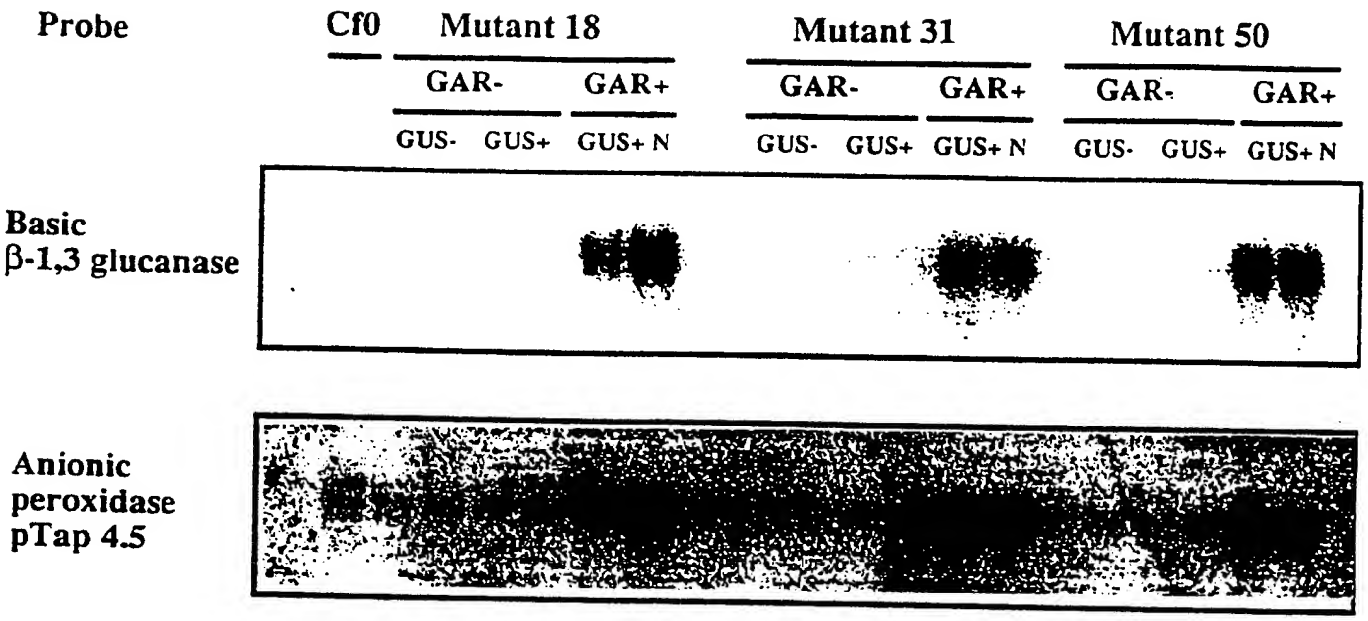


FIGURE 6

FIGURE 7



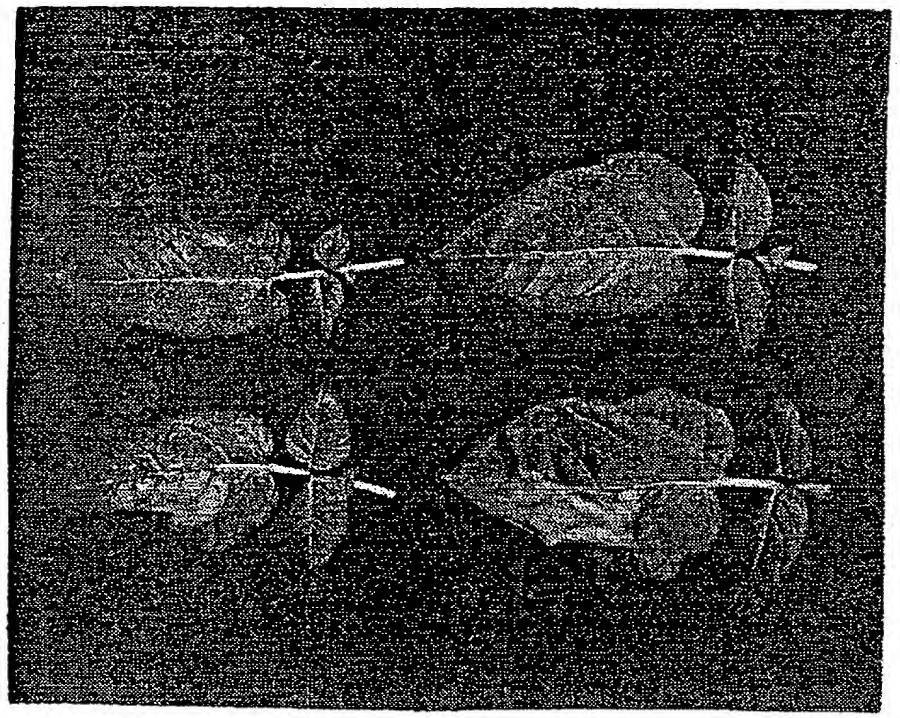


FIGURE 8B

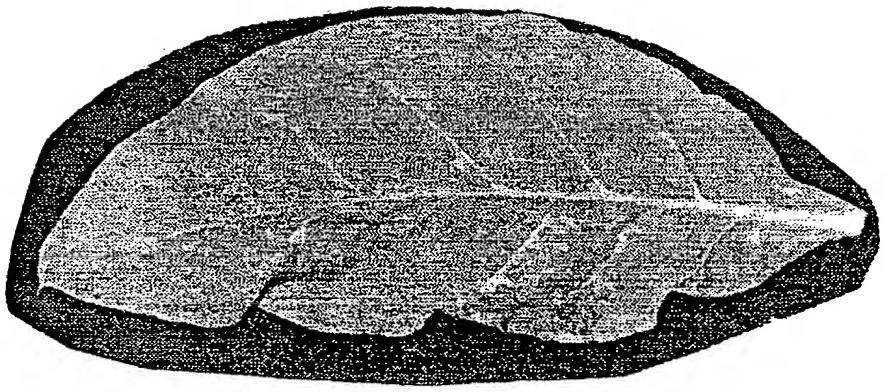


FIGURE 8A

FIGURE 9

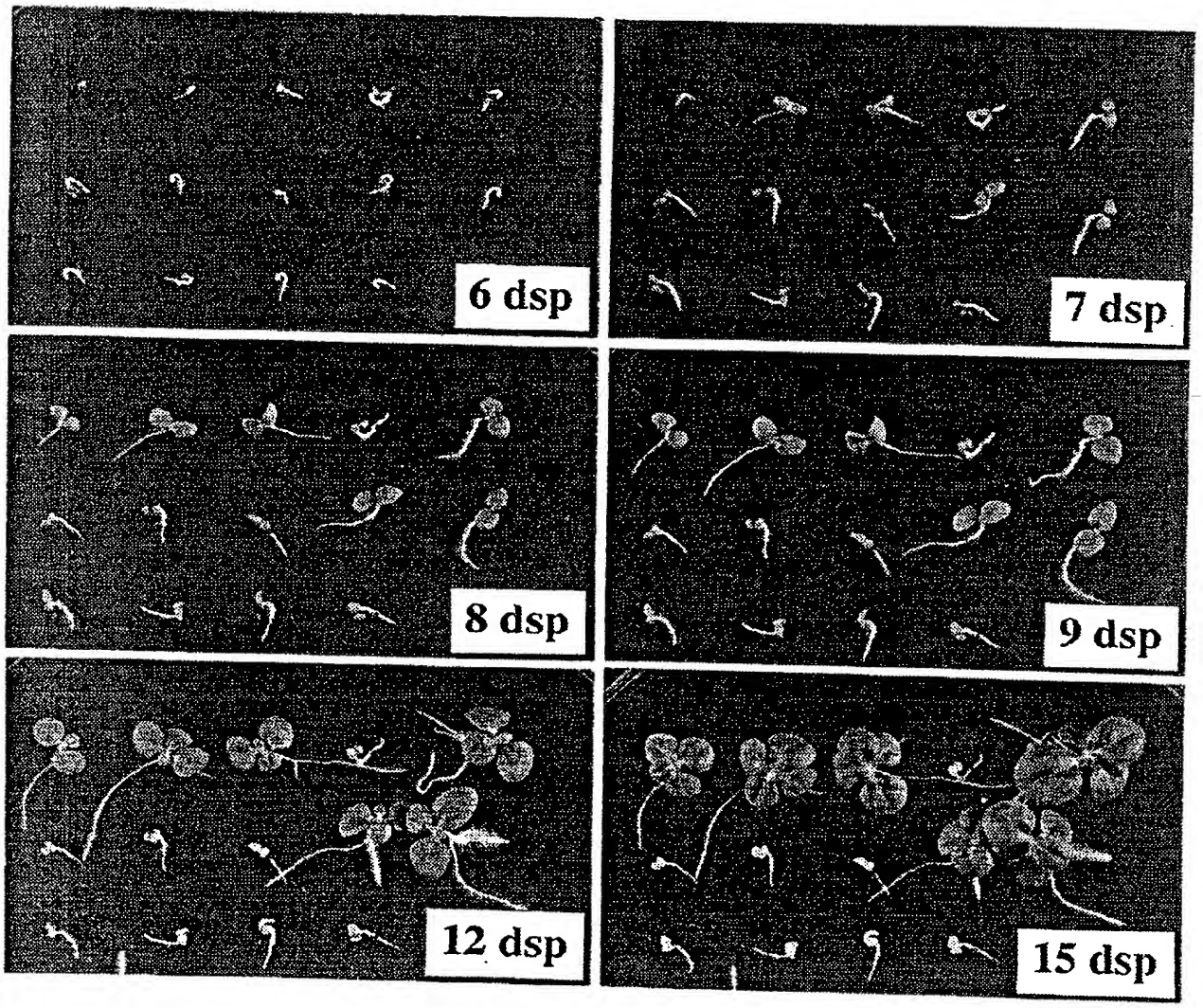
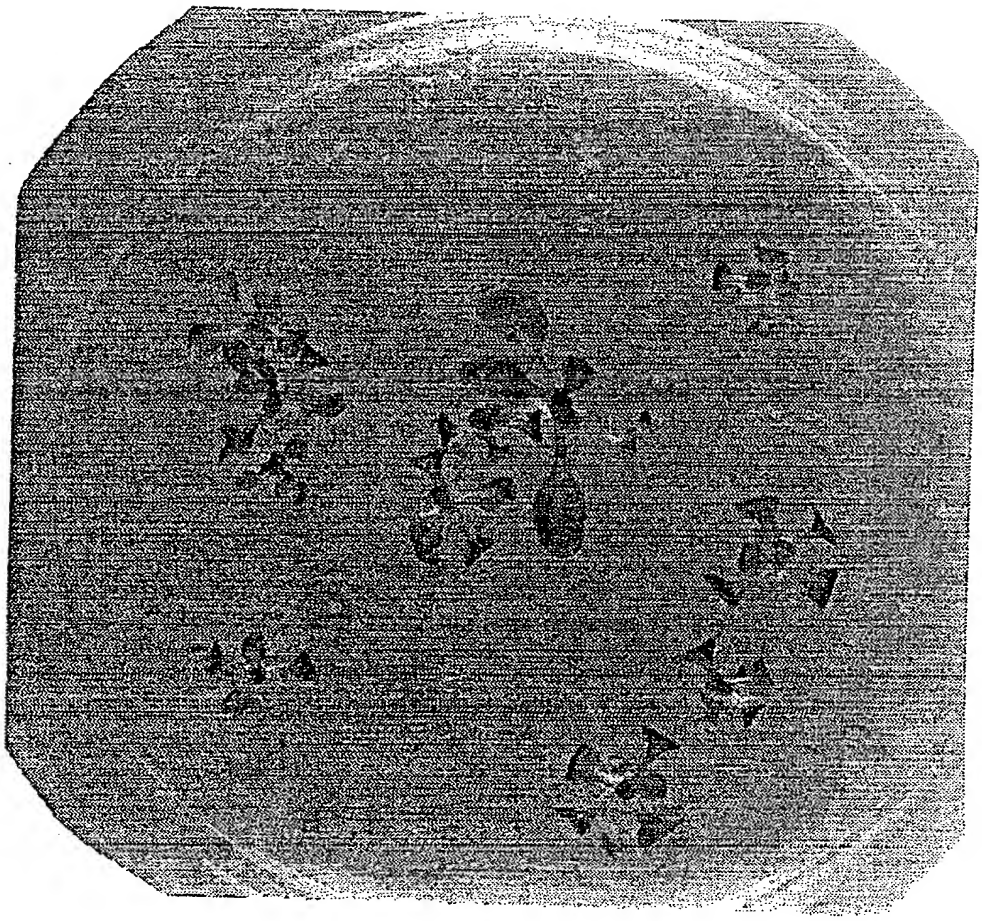


FIGURE 10



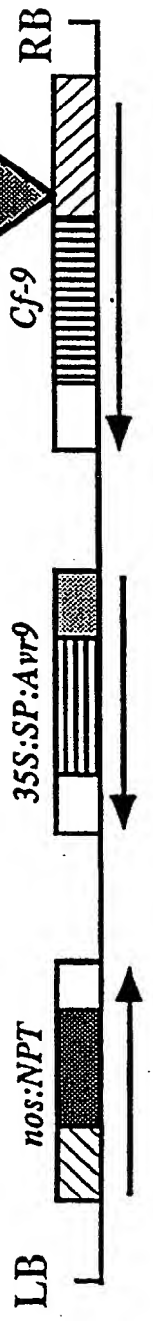
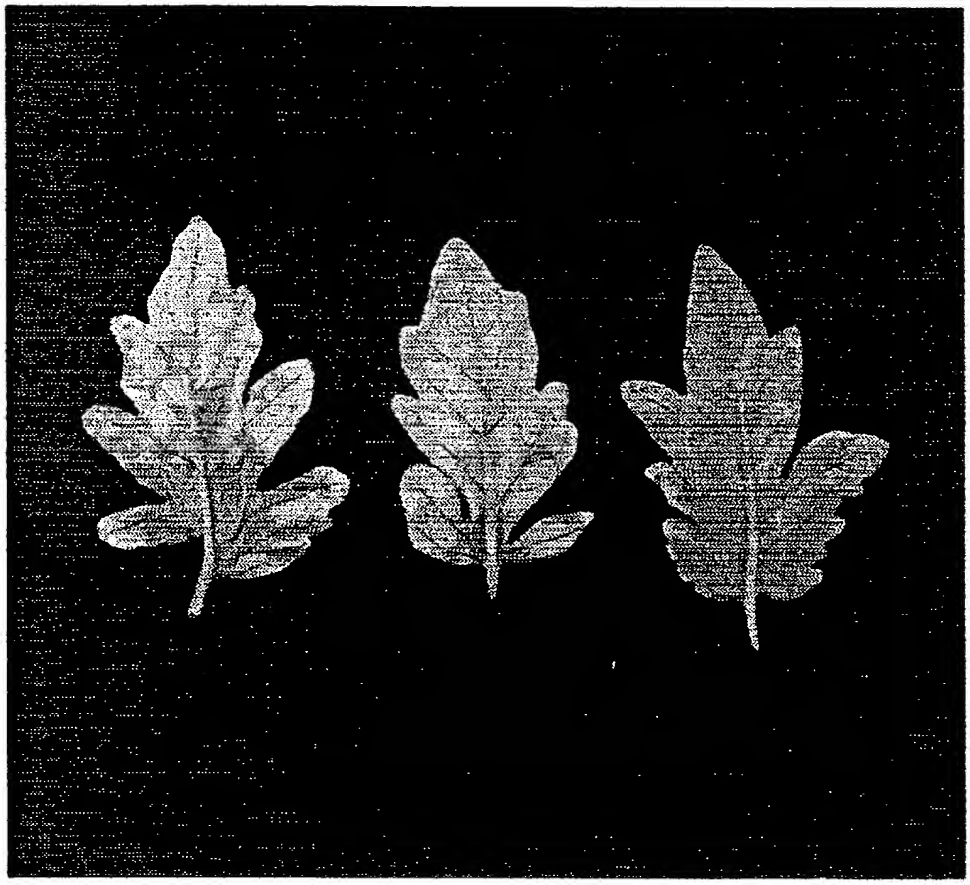


FIGURE 11

Figure 12.



Leaf 1 Leaf 2 Leaf 3

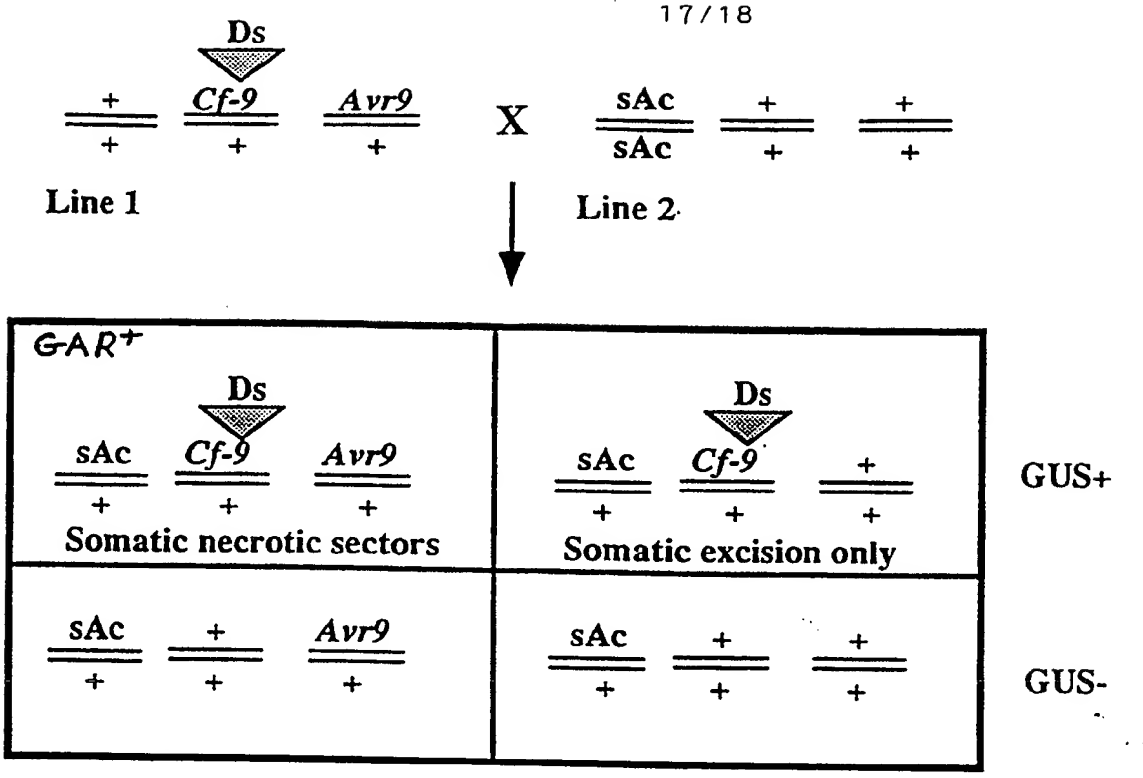
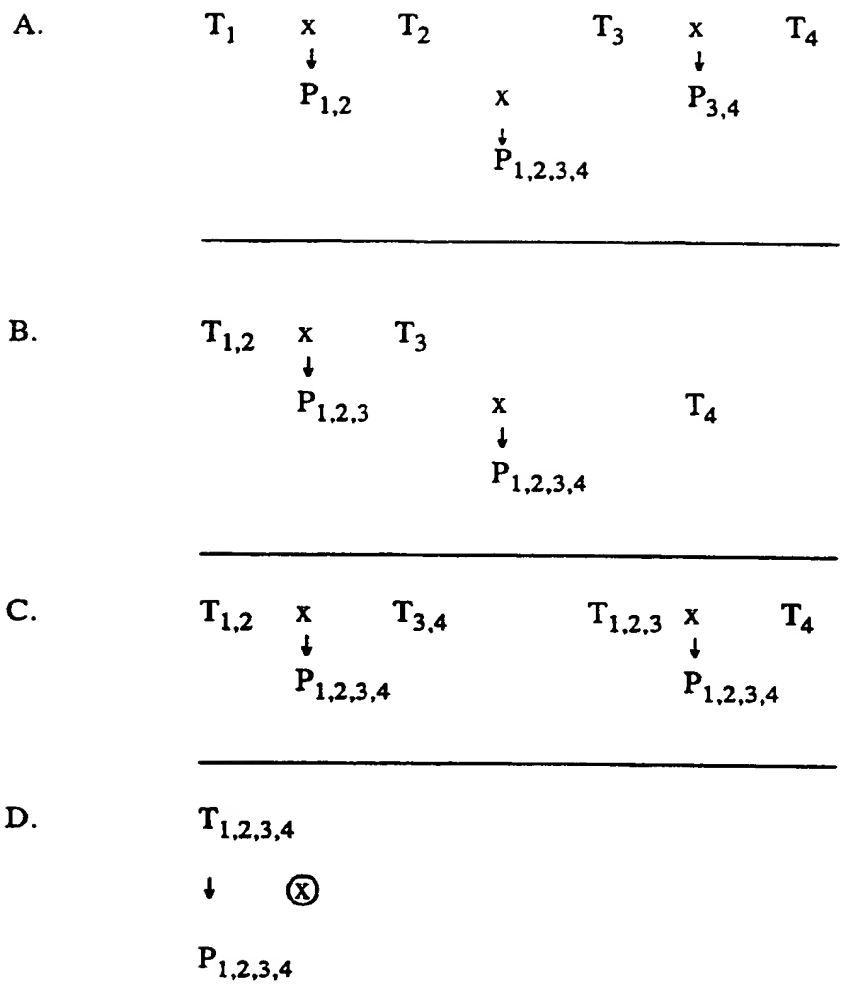

Ds
+ / +
Cf-9 / +
Avr9 / +
X
sAc / sAc
+ / +
+ / +
Line 1
Line 2
GAR+
Ds
sAc / +
Cf-9 / +
Avr9 / +
Somatic necrotic sectors
Ds
sAc / +
Cf-9 / +
+ / +
Somatic excision only
GUS+
sAc / +
+ / +
Avr9 / +
sAc / +
+ / +
+ / +
GUS-

FIGURE 13

FIGURE 14



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(71) Applicant *(for all designated States except US)*: THE GATSBY CHARITABLE FOUNDATION [GB/GB]; 9 Red Lion Court, London EC4A 3EB (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JONES, Jonathan, Dallas, George [GB/GB]; 19 Waverley Road, Norwich, Norfolk NR4 6SG (GB). HAMMOND-KOSACK, Kim, Elizabeth [GB/GB]; 6 Chestnut Hill, Norwich RN4 6NL (GB). JONES, David, Allen [GB/GB]; 139 Greenways, Eaton, Norwich NR4 6PD (GB).

(74) Agents: WALTON, Seán, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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(57) Abstract

Variegated plants have increased pathogen resistance: cells of the plant express a phenotype, which may comprise necrosis and/or a plant defence response, and other cells not expressing this phenotype have increased pathogen resistance. Embodiments of the invention employ various genes, including *Cladosporium fulvum* pathogen resistance genes, which are inactivated, for example as a result of insertion of a transposable genetic element, and then reactivated in plant cells to result in necrosis and/or a plant defence response, leading to increased pathogen resistance. Cells, plants and other compositions of matter are provided comprising various combinations of genes involved in this system.

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INTERNATIONAL SEARCH REPORT

Internatio Application No
PCT/GB 95/01075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29 C12N15/31 C12N5/10 A01N63/02
A01N65/00 A01H5/00

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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 23532 (RIBOZYME PHARMACEUTICALS INC) 25 November 1993 see the whole document ---	1-4,21, 33,43-45
X	WO,A,92 13090 (GEN HOSPITAL CORP) 6 August 1992 see page 11, line 21 - page 12, line 2 see page 33, line 13 - line 20; example 2 ---	1-4,21, 33,43-45
X	WO,A,92 13089 (GEN HOSPITAL CORP ;HARVARD COLLEGE (US)) 6 August 1992 see page 12, line 3 - line 24 see page 35, line 3 - line 10; example 2 ---	1-4,21, 33,43-45
A	WO,A,91 15585 (RIJKSLANDBOUWHOGESCHOOL) 17 October 1991 see the whole document ---	1-56
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Date of the actual completion of the international search	Date of mailing of the international search report
19 October 1995	16. 11. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Maddox, A

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P,X	SCIENCE, vol. 266, 4 November 1994 LANCASTER, PA US, pages 789-793, JONES, D.A., ET AL. 'ISOLATION OF THE TOMATO CF-9 GENE FOR RESISTANCE TO CLADOSPORIUM FULVUM BY TRANSPOSON TAGGING' see page 789, column 3 ---	21-56
P,X	SCIENCE, vol. 268, 5 May 1995 LANCASTER, PA US, pages 661-667, STASKAWICZ, B.J., ET AL. 'MOLECULAR GENETICS OF PLANT DISEASE RESISTANCE' see page 666, column 3 ---	1-56
A	MOL. PLANT MICROBE INTERACT., vol. 4, no. 5, 1991 pages 458-463, CULVER, J.N., ET AL. 'TOBACCO MOSAIC VIRUS ELICITOR COAT PROTEIN GENES PRODUCE A HYPERSENSITIVE PHENOTYPE IN TRANSGENIC NICOTIANA SYLVESTRIS PLANTS' see the whole document ---	1-56
A	MOLECULAR AND GENERAL GENETICS, vol. 239, 1993 BERLIN DE, pages 122-128, WOLTER, M., ET AL. 'THE MLO RESISTANCE ALLELES TO POWDERY MILDEW INFECTION IN BARLEY TRIGGER A DEVELOPMENATAALY CONTROLLED DEFENSE MIMIC PHENOTYPE' see the whole document ---	1-56
P,A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994 WASHINGTON US, pages 10445-10449, HAMMOND-KOSACK, K.E., ET AL 'DEVELOPMENTALLY REGULATED CELL DEATH ON EXPRESSION OF THE FUNGAL AVIRULENCE GENE AVR9 IN TOMATO SEEDLINGS CARRYING THE DISEASE-RESISTANCE GENE CF-9' see page 10449, left column, last paragraph --- -/--	1-56

3

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PCT/GB 95/01075

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	SCIENCE, vol. 262, November 1993 LANCASTER, PA US, pages 1432-1436, MARTIN, G.B., ET AL. 'MAP-BASED CLONING OF A PROTEIN KINASE GENE CONFERRING DISEASE RESISTANCE IN TOMATO' see the whole document	1-56
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio Application No
PCT/GB 95/01075

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9323532	25-11-93	AU-B-	4234293	13-12-93
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		EP-A-	0641384	08-03-95
		JP-T-	7507931	07-09-95
WO-A-9213090	06-08-92	AU-B-	654330	03-11-94
		AU-B-	1234492	27-08-92
		CN-A-	1065682	28-10-92
		EP-A-	0567568	03-11-93
		JP-T-	6504444	26-05-94
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		CN-A-	1065681	28-10-92
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		JP-T-	6507308	25-08-94
		NZ-A-	241311	28-03-95
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